

Identification and characterization of carboxylate transporters in *Corynebacterium glutamicum*

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln
vorgelegt von

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Köln, September 2008

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Tag der Disputation: 25.11.2008

Pilate: „And why did you stir up people in the bazaar, you vagrant, talking about the truth, of which you have no notion? What is truth?“

„The truth is, first of all, that your head aches, and aches so badly that you're having faint-hearted thoughts of death. You're not only unable to speak to me, but it is even hard for you to look at me. And I am now your unwilling torturer, which upsets me...“

*Mikhail Bulgakov
The Master and Margarita*

Abstract

Transport processes play an important role in cellular fluxes due to the impermeability of the plasma membrane for most substrates. The understanding of carboxylate metabolism is of particular interest for fundamental research, approaching the goal of a complete understanding of metabolic pathways. Biotechnological applications benefit from this knowledge, since production processes can be improved by increasing the yield and reducing the production costs. At the beginning of this work, the transport processes contributing to carboxylate uptake and excretion were largely unknown. Here, several carboxylate transporters were identified and characterized and some putative transporters were suggested, thus promoting the global understanding of carboxylate metabolism in the biotechnologically important organism *C. glutamicum*.

The pyruvate importer MctC was identified and proven to be a high-affinity acetate and propionate carrier. It was shown to be indispensable for pyruvate utilization and to contribute substantially to acetate and propionate uptake under natural conditions of low substrate availability. The activity of MctC was found to depend on the carbon source present in the medium and its transcription was regulated by the major regulators of acetate metabolism RamA and RamB. Furthermore, it was shown to be transcribed in an operon with a small membrane protein encoding gene *cgl0832*.

The characterization of spontaneous mutants, which were able to grow on succinate, fumarate, or L-malate, led to the identification of the transporters DccT and DctA. DccT was proven to be a secondary active, Na⁺ dependent C₄-dicarboxylate importer. Its presence promoted aerobic growth on succinate, fumarate, L-malate, and oxaloacetate. DctA was also verified to confer dicarboxylate uptake ability. In contrast to DccT, DctA was shown to depend on the electrochemical proton potential and to accept many structurally different carboxylates as substrates, including the C₆-tricarboxylate citrate, the C₅-dicarboxylate 2-oxoglutarate, the C₄-dicarboxylates succinate, fumarate, L-malate, oxaloacetate, and aspartate, as well as the monocarboxylates glyoxylate and lactate, albeit its substrate affinity was found to be lower than that of DccT.

Several different approaches were undertaken in order to identify carboxylate exporters. Although none of them was successful for the identification of a lactate exporter, a putative succinate exporter was suggested by the characterization of site-directed mutants during oxygen deprived conditions. The analysis of gene expression under pyruvate producing conditions as well as phenotypic analysis of site-directed mutants pointed to several putative pyruvate exporters.

Kurzzusammenfassung

Aufgrund der eingeschränkten Membranpermeabilität für die meisten Substrate, ist deren Transport entscheidend für die Aufrechterhaltung des Metabolismus und damit ein wichtiger Faktor für das Verständnis des Stoffwechsels. Der Transport von Carboxylaten im biotechnologisch bedeutenden Bakterium *C. glutamicum* war bisher weitgehend unbekannt. Ziel dieser Arbeit war es, ein möglichst umfangreiches Verständnis vom Carboxylattransport zu gewinnen, indem bereits vorgeschlagene Carboxylatcarrier charakterisiert und neue Systeme identifiziert werden sollten.

Der Pyruvatimporter MctC wurde identifiziert und biochemische Analysen belegten seine Funktion als hochaffines Acetat- und Propionataufnahmesystem. Es wurde gezeigt, dass es essentiell für die Verwertung von Pyruvat ist und maßgeblich zur Acetat- und Propionataufnahme unter natürlichen limitierten Bedingungen beiträgt. Die Transkription von *mctC* findet im Operon mit *cgl0832* statt und wird von den beiden Hauptregulatoren des Acetatmetabolismus RamA und RamB in Abhängigkeit von der angebotenen Kohlenstoffquelle bestimmt.

Die Charakterisierung von Spontanmutanten, die auf succinat-, fumarat- oder L-malathaltigem Medium isoliert wurden, erlaubte die Identifizierung der beiden Dicarboxylattransporter DccT und DctA. Biochemische Studien zu DccT belegten, dass es ein sekundär aktiver, Na⁺-abhängiger Transporter ist. Durch seine Anwesenheit wird das aerobe Wachstum von *C. glutamicum* auf Succinat, Fumarat, L-Malat und Oxalacetat ermöglicht. Zusätzlich vermittelt auch die Anwesenheit von DctA die Fähigkeit zur Dicarboxylataufnahme. Im Gegensatz zu DccT ist DctA ein protonenabhängiger Transporter, der eine Vielzahl strukturell unterschiedlicher Substrate bindet. Im Vergleich mit DccT zeigte DctA jedoch eine geringere Substrataffinität.

Durch Genexpressionsstudien unter Pyruvat produzierenden Bedingungen sowie durch die phänotypische Charakterisierung einiger ortsgerichteter Mutanten unter O₂ limitierten Bedingungen konnten Transporter identifiziert werden, die möglicherweise am Export von Pyruvat und Succinat beteiligt sind.

Die in dieser Arbeit gewonnenen Daten liefern ein umfangreiches Verständnis der Carboxylataufnahme und -exkretion in *C. glutamicum*. Damit können biotechnologische Anwendungen durch gezielte Eingriffe in die Transportvorgänge maßgeblich verbessert werden, wodurch die Substratausbeute gesteigert und damit die Produktionskosten gesenkt werden können.

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Abbreviations

A	Absorption
a	Year
ADP / ATP	Adenosine-5'-diphosphate / Adenosine-5'-triphosphate
AEP	1-aminoethylphosphinate
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
bp	Base pairs
BSA	Bovine serum albumine
CCCP	Carbonyl cyanide <i>m</i> -chlorophenoxyhydrazone
cdm	Cellular dry matter
cpm	Counts per minute
DAACS	Dicarboxylate/amino acid:cation symporter
DASS	Divalent anion:Na ⁺ symporter
DMSO	Dimethyl sulfoxide
DSM	German Collection of Microorganisms
E	Extinction
EDTA	Ethylendiaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
et al.	et alii (and others)
F	Faraday constant (F = 96.485 coulomb/mole)
G	Gibbs energy
IPTG	Isopropyl-D-thiogalactopyranoside
K _{0.5}	Substrate concentration at which half of V _{max} is reached
kb	Kilo base pairs
K _m	Michaelis-Menten constant
Km ^R	Resistant to kanamycin
LB	Luria Bertani medium
mb	Mega base pairs
MES	2-(N-morpholino)-ethanesulfonic acid
MFS	Major facilitator superfamily
mio	Million
MM	Minimal medium
MOPS	3-(N-morpholino)-propanesulfonic acid
MSTFA	<i>n</i> -methyl- <i>n</i> -trimethylsilyl-trifluoroacetamide
MTP	Microtiter plate

NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
OD	Optical density
P	Phosphate
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PTS	Phosphotransferase system
R	Ideal gas constant ($R = 8.314472 \text{ J x K}^{-1} \text{ x mol}^{-1}$)
RND	Resistance-Nodulation-Cell Division transporter superfamily
Rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecylsulphate
SSS	Solute:Sodium Symporter
T	Absolute temperature in Kelvin (K)
t	Tons
TCA	Tricarboxylic acid cycle
Tn	Transposon
TRAP	Tripartite ATP-independent periplasmic transporter
Tris	2-amino-hydroxymethylpropane-1,3-diol
v/v	Volume/volume
V_{\max}	Maximal velocity
w/v	Weight per volume
WT	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside
ΔpH	pH-gradient
$\Delta\Psi$	Membrane potential

1 Introduction

1.1 *Corynebacterium glutamicum*

Corynebacterium glutamicum is a Gram-positive, non-motile, aerobic, rod-shaped soil bacterium which grows on a broad substrate spectrum, covering sugars, amino acids and carboxylates. The *Corynebacteriaceae* family belongs to the order *Actinomycetales* / suborder *Corynebacterineae* together with the *Mycobacteriaceae* and four other families, all of which have a DNA of high G+C content and a peculiar cell wall containing mycolic acids (Stackebrandt *et al.*, 1997). Species of these families were isolated from human clinical samples, animals, feces, soil, vegetables and fruits, or animal fodder. Among the *Corynebacterineae* are several pathogenic species as *C. diphtheriae*, *Mycobacterium tuberculosis* or *M. leprae*. They are causative agents of severe diseases and research work on a non-pathogenic model organism like *C. glutamicum* is therefore of high interest. Moreover, research focused on the genus *Corynebacterium* since the discovery of the glutamate producing species *C. glutamicum* (Kinoshita *et al.*, 1957). Presently, *C. glutamicum* is used for the production of 1.5 mio t/a L-glutamate, 0.5 mio t/a L-lysine and minor amounts of L-threonine, glutamine, alanine, isoleucine, nucleotides, and vitamins (Leuchtenberger, 1996; Hermann, 2003). Due to the ongoing research because of its biotechnological importance, the knowledge on *C. glutamicum* is constantly growing. The complete genome of *C. glutamicum* has been sequenced and annotated repeatedly (Kalinowski *et al.*, 2003; Ikeda and Nakagawa, 2003) and the genomes of *C. glutamicum* R, *C. efficiens*, *C. diphtheriae*, *C. jeikeium*, and *C. urealyticum* are sequenced as well. With this and a broad tool kit for genetic manipulation research approaches at the genomic level have become possible. Broad transcriptome, proteome and metabolome analyses were undertaken under various conditions (Dominguez *et al.*, 1998; Krömer *et al.*, 2004; Silberbach *et al.*, 2005; Polen *et al.*, 2007; Qi *et al.*, 2007; Wendisch, 2008). More detailed investigations were performed on the central carbon metabolism and its regulation (Yokota and Lindley, 2005; Eikmanns, 2005; Arndt and Eikmanns, 2008). Playing a decisive part in production efficiency, transport processes regarding mainly sugar import (Moon *et al.*, 2005) and amino acid im- and export (Marin and Krämer, 2007) were investigated and the corresponding transporter encoding genes were identified.

Due to the properties and the application of *C. glutamicum* in biotechnology, it represents – besides *Escherichia coli* and *Bacillus subtilis* – one of the major model organisms in microbiology.

1.2 Carboxylate metabolism

As a widely distributed organism, *C. glutamicum* is able to grow on many different carbohydrates, alcohols, amino and carboxylic acids as single or combined sources of carbon and energy (Liebl, 2001). The utilization of most carboxylates requires the presence of an at least partially functional tricarboxylic acid cycle (TCA) (Fig. 1.1). During growth on non-carbohydrate substrates, this is the starting point for gluconeogenesis. It is accomplished by the oxaloacetate decarboxylating reaction to PEP performed by PEP carboxykinase (PEPCK). Although the presence of oxaloacetate decarboxylase (ODx) was demonstrated in *C. glutamicum*, its participation in the *in vivo* C₄-decarboxylating flux has not been observed yet (Jetten and Sinskey, 1995; Petersen *et al.*, 2000). NADPH generation during growth on substrates other than glucose was proposed to proceed via the NADP(H) dependent malic enzyme catalyzing the reversible decarboxylation of malate to pyruvate (Dominguez *et al.*, 1998). Depending on the present carboxylate, several further reactions are required for proper growth. Thus, acetate utilization requires its activation by acetate kinase (AK) and subsequent transfer to CoA by phosphotransacetylase (PTA), resulting in acetyl-CoA formation. For anaplerosis, the glyoxylate cycle supplies the TCA with malate. Malate can be converted to oxaloacetate either by the endergonic reaction catalyzed by the NAD⁺ dependent malate dehydrogenase (MDH) or by the exergonic reaction catalyzed by the membrane-associated menaquinone (Q) dependent malate:quinone oxidoreductase (MQO) concomitantly fueling electrons into the respiratory chain. The presence of a functional MQO was shown to be essential for growth on minimal medium whereas the absence of MDH caused no obvious phenotype (Molenaar *et al.*, 2000).

Growth on propionate has also been observed for *C. glutamicum*, albeit only after a long lag phase (Claes *et al.*, 2002). Its utilization requires the presence of a functional 2-methylcitrate-cycle, which converts propionate and oxaloacetate to pyruvate and succinate. The activation of propionate is usually performed by acetyl-CoA synthetase, but since there was no indication for activity of acetyl-CoA synthetase in *C. glutamicum* during growth on propionate, it was suggested to occur via acetate kinase and phosphotransacetylase (Reinscheid *et al.*, 1999; Claes *et al.*, 2002). Moreover, propionyl-CoA can be derived from the degradation of odd-numbered fatty acids. The accumulation of intermediates of the 2-methylcitrate-cycle was shown to have bacteriostatic consequences. So, propionyl-CoA was shown to inhibit pyruvate dehydrogenase in *Rhodobacter sphaeroides* (Maruyama and Kitamura, 1985), and the accumulation of 2-methylcitrate repressed *Salmonella typhimurium* growth due to inhibition of isocitrate dehydrogenase (Horswill *et al.*, 2001). Growth inhibition due to 2-methylcitrate accumulation

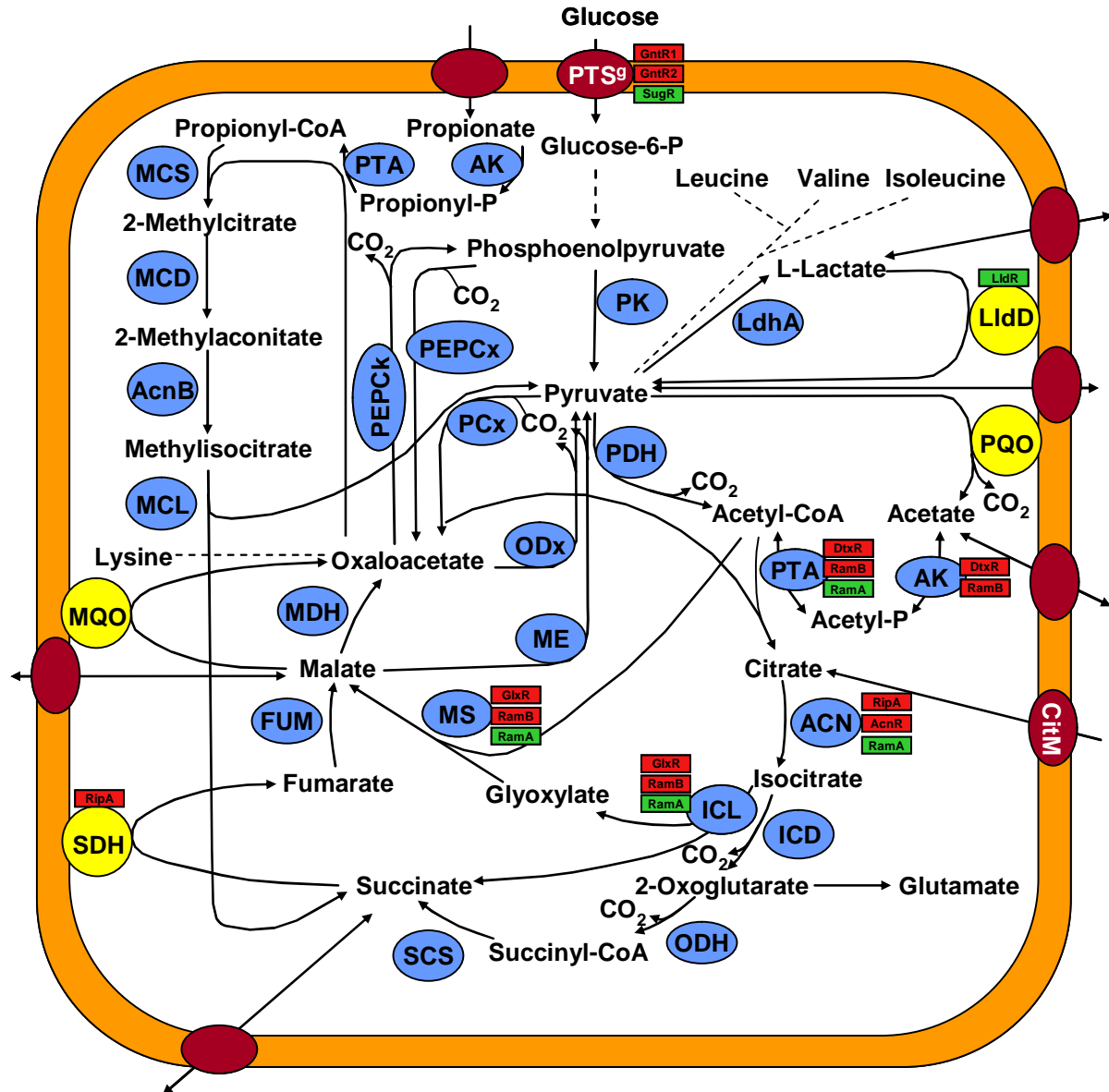


Fig. 1.1 Overview on the metabolism of selected carboxylates in *C. glutamicum*, in particular the reactions and transcriptional regulation of the tricarboxylic acid cycle, the glyoxylate bypass, the 2-methylcitrate cycle, the PEP-pyruvate-oxaloacetate node and the relevant metabolism of acetate, L-lactate and propionate. Soluble enzymes are shown in blue, membrane associated menaquinone dependent enzymes in yellow and identified or postulated transporter proteins in dark red. Transcriptional regulators repressing the expression of the genes encoding the indicated enzymes are indicated by red squares, the inducers are indicated by green squares. RamA/RamB regulation of *pta-ackA* transcription during propionate utilization has been omitted. Dotted lines indicate that several reactions were omitted between the respective metabolites.

AK: acetate kinase, ACN: aconitase, AcnB: aconitase B, CitM: citrate transporter, Fum: fumarase, ICD: isocitrate dehydrogenase, ICL: isocitrate lyase, LdhA: NADH-dependent lactate dehydrogenase, LldD: menaquinone-dependent lactate dehydrogenase, MCD: 2-methylcitrate dehydratase, MCS: 2-methylcitrate synthetase, MCL: 2-methylcitrate lyase, MDH: malate dehydrogenase, ME: malic enzyme, MS: malate synthase, MQO: malate:quinone oxidoreductase, ODH: 2-oxoglutarate dehydrogenase, ODX: oxaloacetate decarboxylase, PCx: pyruvate carboxylase, PDH: pyruvate dehydrogenase, PEPCK: PEP carboxykinase, PEPCx: PEP carboxylase, PK: pyruvate kinase, PTA: phosphotransacetylase, PTS^g: glucose specific PTS system, PQA: pyruvate:quinone oxidoreductase, SCS: succinyl-CoA synthetase, SDH: menaquinone dependent succinate dehydrogenase. (Adapted from Eikmanns, 2005).

was also observed for *C. glutamicum* (Plassmeier *et al.*, 2007), although its action was not addressed.

Lactate metabolism leads directly to pyruvate via oxidation by the menaquinone dependent membrane-associated lactate dehydrogenase LldD. The NADH dependent soluble lactate dehydrogenase LdhA catalyzes the exergonic reduction of pyruvate to lactate under limited respiration conditions and lactate is subsequently released into the medium. Another consequence of a decelerated TCA under limited respiration conditions is the carboxylation of PEP to oxaloacetate and its subsequent reduction to malate. Malate is either decarboxylated to pyruvate, yielding 1 NADPH or subsequently reduced to succinate, which is released into the medium (Inui *et al.*, 2004). Furthermore, acetyl-CoA is converted to acetate by PTA and AK and also released into the medium. In addition, the analysis of the *C. glutamicum* genome revealed the presence of a peripheral pyruvate:quinone oxidoreductase, which directly decarboxylates pyruvate to acetate upon reduction of menaquinone (Bott and Niebisch, 2005).

In addition to its role in anabolic reactions, the TCA cycle is decisive for the generation of reducing equivalents for respiration and precursors for various biosynthetic reactions such as the synthesis of lysine and glutamate, which is crucial for the use of *C. glutamicum* in biotechnology. The continuous loss of intermediates withdrawn for other reactions requires the constitutive replenishment of the TCA for its proper function. In *C. glutamicum*, this is accomplished by 90% through carboxylation of pyruvate by pyruvate carboxylase (PCx) and to 10% through carboxylation of phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxylase (PEPCx) during growth on glucose (Petersen *et al.*, 2000). During growth on carboxylates such as acetate or propionate, the TCA is replenished with the aid of the glyoxylate or 2-methylcitrate cycle.

As indicated in Fig. 1.1, irrespective of the carbon source, the decisive fine-tuning of pyruvate decarboxylation to acetyl-CoA supplying the TCA, the anaplerotic C₃-carboxylation for TCA replenishment and the opposite gluconeogenic reaction leading to pyruvate or PEP takes place at the PEP-pyruvate-oxaloacetate node (Sauer and Eikmanns, 2005), underlying the central role of pyruvate in the versatile metabolism of carboxylates.

1.3 Regulation of carboxylate metabolism

As a soil organism, *C. glutamicum* frequently has to cope with changing natural conditions including the external pH, osmolarity, temperature, oxygen concentration and nutrient availability. Quick acclimatization to new conditions is therefore of high importance in nutrient competition or for the maintenance of energy budget and homeostasis. As depicted in Fig. 1.1, *C. glutamicum* both utilizes and produces the fermentative end-products acetate and lactate. Uncontrolled fluxes would result in futile cycles and therefore require tight regulation. The complex interaction of reactions at the PEP-pyruvate-oxaloacetate node

must be quickly adaptable to balance the carbon and redox state of the cell. In contrast to other bacteria, *C. glutamicum* rarely shows diauxic growth when cultivated on a substrate mixture. So far, preferential utilization of glucose has been observed only in combination with glutamate or ethanol, and acetate was preferred to ethanol (Krämer *et al.*, 1990; Arndt *et al.*, 2007; Arndt and Eikmanns, 2007). Other substrates such as L-lactate, pyruvate, acetate, propionate and several others were metabolized concomitantly. Data on global regulators as CRP or the CcpA dependent carbon catabolite repression system such as in *E. coli* or *B. subtilis* (Cocaign *et al.*, 1993; Wendisch *et al.*, 2000; Claes *et al.*, 2002) is scarcely found. A CRP-like cAMP binding regulator DRP was recently found to regulate the acyl-CoA carboxylase encoding *dtsR1*, but further information on global carbon control is required (Kimura, 2002).

So far, eight different transcriptional regulators were found to be primarily involved in the regulation of carbon metabolism (Fig. 1.1). The expression of *ptsG* is activated by the functionally equivalent GntR1 and GntR2 transcriptional regulators in the absence of gluconate (Frunzke *et al.*, 2008) and repressed by SugR in the presence of acetate, pyruvate and citrate (Engels and Wendisch, 2007). The fluxes through glycolysis are supposed to be influenced by the ratio of ATP/AMP at the level of pyruvate kinase (Jetten *et al.*, 1994) while the TCA rate-controlling citrate synthase is inhibited by ATP, aconitate and isocitrate (Eikmanns *et al.*, 1994). In addition, it was shown that the expression levels of genes for glycolysis and TCA cycle changed with respect to the applied carbon source (Hayashi *et al.*, 2002; Muffler *et al.*, 2002; Gerstmeir *et al.*, 2003; Han *et al.*, 2007). In particular, the expression of the aconitase encoding *acn* gene was shown to be repressed by AcnR during growth on citrate, acetate or propionate (Krug *et al.*, 2005). Moreover, *acn* and *sdhCAB* encoding aconitase and succinate dehydrogenase both contain iron-sulfur clusters and are regulated by DtxR and RipA, which control iron homeostasis (Wennerhold *et al.*, 2005; Wennerhold and Bott, 2006). Post-transcriptional regulation of the TCA is realized at least at the level of 2-oxoglutarate dehydrogenase (ODH) activity, which is increased in the presence of glutamine due to the phosphorylation state of OdhI. This is accomplished by the Ser/Thr protein kinase PknG and the Ppp phosphatase (Niebisch *et al.*, 2006; Schultz *et al.*, 2007; Bott, 2007).

Utilization of acetate requires the activity of acetate kinase, phosphotransacetylase, isocitrate lyase and malate synthase. The expression of the corresponding genes *ackA*, *pta*, *aceA*, and *aceB* is regulated by the concerted action of RamA and RamB. Additionally, *aceA* and *aceB* are negatively regulated by GlxR in the presence of cAMP, which is positively correlated to the presence of glucose in the medium (Kim *et al.*, 2004; Gerstmeir *et al.*, 2004; Cramer *et al.*, 2006). In the absence of iron, the expression of *pta* and *ackA* is additionally repressed by DtxR (Wennerhold and Bott, 2006).

L-lactate consumption requires the presence of the membrane associated lactate dehydrogenase LldD, whose expression is repressed in the presence of glucose by LldR (Stansen *et al.*, 2005; Georgi *et al.*, 2008). Further gluconeogenic enzymes such as PEP carboxykinase and fructose-1,6-bisphosphatase, which are regulated by fructose-1-phosphate, PEP, ATP, AMP and phosphate are required for growth on L-lactate. At least the PEP carboxykinase encoding *pck* was shown to be differentially expressed, although the regulator has not been identified yet (Jetten and Sinskey, 1993; Riedel *et al.*, 2001; Rittmann *et al.*, 2003).

Propionate metabolism requires the effective action of the 2-methylcitrate cycle, since the accumulation of 2-methylcitrate has toxic effects on the cell (Plassmeier *et al.*, 2007). Although an induction of the *prpD2B2C2* operon upon growth on propionate has been observed, its regulation remains elusive. A faint hint is the induction of *dtsR1* and *dtsR2*, encoding putative carboxyltransferase domains of acyl-CoA carboxylase upon growth on acetate and propionate (Hüser *et al.*, 2003). *dtsR1* was shown to be under the control of the DRP regulator, suggesting regulation of propionate utilization on global regulatory level.

Although the regulation of carboxylate metabolism is not completely unravelled yet and further regulators are sure to be discovered, the already evolving picture of complex regulation of carboxylate utilization underlines its importance for the cell. The understanding of the regulatory network offers the opportunity to re-direct fluxes towards the desired products in biotechnological applications, thus increasing production efficiency. On the other hand, global regulatory mechanisms controlling basic cellular processes are likely to function similarly in pathogenic relatives of *C. glutamicum*, thus being potential targets for drug development.

1.4 Carboxylate transport

Cellular substrate fluxes are determined not only by anabolic and catabolic reactions but also by the availability (i.e. influx) and loss (i.e. efflux) of the respective compounds. Thus, transporters play a crucial part in metabolism due to the impermeability of the cell membrane for most substrates. Different mechanisms enable the cell to take up or excrete substrates (Krämer, 1999):

- 1) facilitated diffusion through carriers
- 2) primary transport through ABC-type transporters, which rely on ATP
- 3) secondary transport either in symport or antiport with an electrochemically relevant ion, thus making use of the electrochemical potential
- 4) group translocation through the PTS system, which transfers a phosphoryl group from PEP to the translocated substrate

Especially in the case of organic acids, the idea of diffusion through the cytoplasmic membrane of the protonated form has been a matter of debate for a long time (Kell *et al.*,

1981; Kaim and Dimroth, 1999) and is increasingly challenged since diffusion was not observed at physiological pH and several carboxylate carriers were identified (Janausch *et al.*, 2001; 2002). Of these, mainly the C₄-dicarboxylates transporters were thoroughly studied at the genetic and biochemical level. The corresponding carriers can be grouped into 5 different families according to protein sequence similarities. The DctA family (subgroup of DAACS (dicarboxylate/amino acid:cation symporter)) was found in aerobic and facultative anaerobic bacteria and serves for uptake of C₄-dicarboxylates during aerobic growth (Asai *et al.*, 2000; Janausch *et al.*, 2002). The best analyzed system is DctA from *E. coli*. It was shown to import succinate, fumarate, malate, and aspartate in a H⁺ dependent manner and was required for orotate utilization (Kay and Kornberg, 1971; Gutowski and Rosenberg, 1975; Baker *et al.*, 1996). Furthermore, *E. coli* and some other (facultative) anaerobic bacteria possess DcuAB and DcuC type transporters for dicarboxylate transport. DcuAB was found in bacteria capable of anaerobic fumarate respiration and is mainly used for fumarate:succinate antiport but also for the uptake of C₄-dicarboxylates (Six *et al.*, 1994; Engel *et al.*, 1992; 1994). Additionally, DcuA was also suggested to catalyze succinate and fumarate uptake at aerobic conditions, thus complementing DctA (Golby *et al.*, 1998). DcuC type transporters were found to be important for C₄-dicarboxylate transport exclusively at anaerobic conditions (Zientz *et al.*, 1996; 1999).

Another class of C₄-dicarboxylate transporters are members of the DASS family (divalent anion:Na⁺ symporter), which are mostly found in eukaryots (Palmieri, 1994), but also in *Staphylococcus aureus*, where SdcS was shown to import C₄-dicarboxylates in a Na⁺ dependent manner (Hall and Pajor, 2005). CitT like transporters, which constitute a subgroup within the DASS family, were found in *E. coli*, *B. subtilis* and *C. glutamicum* (Pos *et al.*, 1998; Warner and Lolkema, 2002; Polen *et al.*, 2007). In *E. coli*, this transporter is essential for anaerobic growth on citrate, exchanging citrate with succinate, fumarate or tartrate. In *C. glutamicum*, only Mg²⁺ dependent citrate uptake was suggested to occur via CitM but there is no evidence for C₄-countertransport with citrate.

Moreover, the TRAP (tripartite ATP-independent periplasmic) type transporter importing C₄-dicarboxylates are found in Gram-negative bacteria and archaea and are predicted to exist in *C. glutamicum* according to its genome annotation. This kind of secondary carrier is peculiar due to the presence of a periplasmic solute-binding protein, which is similar to ABC-type transporters, but ATP independent. The best characterized representative of this class is DctPQM from *Rhodobacter capsulatus* (Forward *et al.*, 1997; Kelly and Thomas, 2001). Its periplasmic subunit was found to bind pyruvate, 2-oxobutyrates and a broad range of aliphatic monocarboxylic 2-oxoacid anions (Thomas *et al.*, 2006). However, in *E. coli*, the C₅-2-oxodicarboxylate 2-oxoglutarate is imported exclusively by the H⁺ dependent KgtP carrier (Seol and Shatkin, 1991; 1992).

Monocarboxylate transporters are members of the 2-hydroxycarboxylate transporter (2-HCT) family, the solute:sodium symporter (SSS) family, the $\text{Na}^+:\text{H}^+$ antiporter (NhaC) family, and the major facilitator superfamily (MFS). Most carboxylates are imported in symport with H^+ , as was shown for the acetate and propionate importer ActP from *E. coli* (Gimenez *et al.*, 2003) and the lactate, acetate and pyruvate importer MctP from *Rhizobium leguminosarum* (Hosie *et al.*, 2002). Some transporters catalyze antiport of fermentative reactants and products. This was observed for transporters from lactic acid bacteria such as CitP from *Leuconostoc mesenteroides* exchanging citrate with lactate (Marty-Teyssset *et al.*, 1995) and MleP exchanging malate with lactate in *Lactococcus lactis* (Bandell *et al.*, 1998). Moreover, a citrate:acetate exchange system was identified in *Klebsiella pneumoniae* which is induced during anaerobic citrate fermentation (Kästner *et al.*, 2002). MaeN, being a member of the NhaC family was also shown to catalyze the electrogenic exchange of malate²⁻ and 2H^+ with Na^+ and lactate⁻ in *B. subtilis* (Wei *et al.*, 2000).

Export of carboxylates without the antiport of another substrate has also been observed in different bacteria. The widely used acetate producer *Acetobacter acetii* exports acetate most likely via a primary active ABC transporter with AatA representing the ATP-binding subunit (Nakano *et al.*, 2006), and probably also via a secondary active system (Matsushita *et al.*, 2005). Lactate export was also shown to be dependent on the electrochemical proton gradient in *Streptococcus cremoris* and *Saccharomyces cerevisiae* (Otto *et al.*, 1980; van Maris *et al.*, 2004a), but the respective transport system has not been identified yet.

With this background of information, data on carboxylate transport in *C. glutamicum* is surprisingly scarce. *C. glutamicum* has been estimated to contain 236 different transport proteins. Most of them are predicted secondary (54.2%) and primary transporters (38.6%). Channels (2.5%) and group translocators (2.5%) are less numerous and diverse, while 1.7% of the predicted carriers could not be classified (Ren *et al.*, 2007). Moreover, 183 further proteins with unknown function are predicted to have at least 3 transmembrane helices and thus might function as a transporter as well. According to the predicted substrate specificity, *C. glutamicum* possesses 31 transporters with predicted substrate specificity for amino acids and derivatives, 53 for inorganic molecules, 14 for sugars, 14 for carboxylates, and 40 for drugs and toxins (Winnen *et al.*, 2005). However, *in silico* predictions of substrate specificity are rarely correct and need to be proven biochemically. In spite of these bioinformatic predictions, the vast majority of the biochemically or genetically characterized transporters refer to sugar or amino acid transport (Moon *et al.*, 2005; Marin and Krämer, 2007). However, only little is known on the transport of carboxylates, although biochemical data suggest the presence of transport systems. Active H^+ dependent import of acetate and Na^+ dependent succinate uptake was shown by Ebbighausen *et al.* (1991a,b), although the corresponding transport system was not identified then. Furthermore, the export of lactate, succinate, acetate and pyruvate has been observed suggesting the presence of the

respective transporter (Inui *et al.*, 2004; Blombach *et al.*, 2007a). The related *C. diphtheriae* and *C. pseudotuberculosis* were shown to produce major amounts of propionate and formate in addition to acetate, and *C. kutscheri* was also observed to excrete minor amounts of oxaloacetate (Reddy and Kao, 1978), but no genetic data is available on these carriers. On the other hand, transcriptional profiling suggested the presence of two citrate importers CitM and TctABC (Polen *et al.*, 2007) and an L-lactate permease (Stansen *et al.*, 2005) although biochemical evidence is lacking. A recent breakthrough was the identification of a pyruvate importer, although a detailed biochemical characterization was not accomplished then (Ballan, 2007).

In summary, there is no concerted biochemical and genetic evidence for any carboxylate carrier in *C. glutamicum*. Especially for this biotechnologically relevant organism, the understanding of carboxylate transport would open the door to numerous biotechnological applications.

1.5 Industrial production and utilization of carboxylates

Microbial production of bulk chemicals using renewable substrates has become increasingly important for industry, especially considering the growing oil prices. Many processes have already become possible due to the discovery of new species or to designed metabolic engineering of already known strains. For example, *C. glutamicum* was discovered in a screen for glutamate producing organisms (Kinoshita *et al.*, 1957) and was turned into an L-lysine producing strain mainly by the insertion of a feedback resistant aspartate kinase, by concomitant increase of the amount of the anaplerotic pyruvate carboxylase, and homoserine dehydrogenase (Kelle *et al.*, 2005). Optimized biotechnological processes are frequently cheaper than chemical production of a compound since waste biomass as starch and molasses can be utilized as carbon source. Additionally, the biotechnological production of one stereoselective compound also reduces production costs due to the needless separation of the enantiomers. Thus, pyruvate production by dehydration and decarboxylation of tartaric acid costs 8.650 \$/t but only 1.255 \$/t by fermentation with *Torulopsis glabrata* (Li *et al.*, 2001). Today, the fermentative production of amino acids, alcohols, organic acids, vitamins, and antibiotics constitutes about 5% of the total market volume with a constantly increasing share (Hatti-Kaul *et al.*, 2008).

Organic acids play an important role in food, medical and technical industry. Nearly every organic acid of the central metabolism is used as a food additive, in pharmaceuticals or in the production of different solvents, resins, or plastics (table 1.1). Of these, acetate, citrate, and lactate have become most important, though the production of other carboxylates has occupied a solid niche and plays an important part in economy. Acetate is used for the production of polymers as vinylacetate or in solvents as ethylacetate, and also as a food preservative, condiment, and acidulant. Citrate has gained importance as a preservative in

foods and beverages, but is also used in water softeners and cathartics. Lactate is also a food preservative and flavour additive and is increasingly important for the production of polymers, in dyeing, textile printing, leather tanning, lacquers, plastics, and solvents. Malate and succinate are used for food flavouring and succinate is becoming important as a surfactant, detergent, and ion chelator. Fumarate can be used as a substitute for tartaric acid in beverages, and is also utilized for manufacture of resins, paints, varnishes, polyhydric alcohols, inks, and for the treatment of the skin disease psoriasis. Propionate is utilized as a food preservative and in the modified form of α -hydroxy-propionate for the treatment of sinusitis. Pyruvate is mainly used as starting material for pharmaceuticals such as L-DOPA, as a dietary agent, and also for the synthesis of polymers and cosmetics.

Table 1.1 Organic acid production and utilization. The annual production in tons per year is given for the sum of chemical and biotechnological production of a given substrate. n. p. = no biotechnological production on industrial scale reported (adapted from Sauer *et al.*, 2008; * from Li *et al.*, 2001).

Organic acid	Production [t/a]	Organism used	Application (examples)
Acetic acid	7.000.000	<i>Saccharomyces</i> and <i>Acetobacter</i>	polymers, solvents, food
Lactic acid	150.000	<i>Lactobacillus</i> spp.	polymers, food
Propionic acid	130.000	<i>Propionibacterium acidipropionici</i>	food and pharmaceuticals
Fumaric acid	12.000	n.p.	polymers, food and pharmaceuticals
Malic acid	10.000	n.p.	food
Succinic acid	16.000	<i>Actinobacillus succinogenes</i> , <i>E. coli</i> , <i>Mannheimia succiniproducens</i> , <i>Anaerobiospirillum succiniciproducens</i>	polymers
Citric acid	1.600.000	<i>Aspergillus niger</i> , <i>Yarrowia lipolytica</i>	food
Pyruvic acid*	>400	<i>Torulopsis glabrata</i>	pharmaceuticals

Several organisms have gained importance as industrial producers of organic acids. Acetate is standardly produced in a two-step reaction, in which *Saccharomyces* strains convert sugars to ethanol, which is subsequently oxidized to acetate by *Acetobacter* strains. Alternatively, an aerobic one-step reaction was developed using an engineered *E. coli* W3110 strain, which reached 75% of the maximal yield on glucose. This was achieved by elimination of fermentative pathways, oxidative phosphorylation and interruption of the TCA, mainly by interference with genes encoding lactate dehydrogenase, pyruvate formate lyase, fumarate reductase, alcohol dehydrogenase, ATPase subunits, and 2-oxoglutarate dehydrogenase (Causey *et al.*, 2003). Citrate production is performed traditionally with the filamentous fungus *Aspergillus niger*, or alternatively by the yeast *Yarrowia lipolytica*, which can utilize n-paraffins or fatty acids as a carbon source (Berovic and Legisa, 2007; Crolla and Kennedy, 2004; Papanikolaou *et al.*, 2006). Lactate is produced by *Lactobacillus*

strains, but a big drawback for industry is their requirement for complex media composition. Alternatively, the fungus *Rhizopus oryzae*, a wine yeast or engineered *E. coli* strains lacking pyruvate formate lyase, D-lactate dehydrogenase and additionally expressing L-lactate dehydrogenase can be utilized (Maas *et al.*, 2006; Saitoh *et al.*, 2005, Dien *et al.*, 2002). The presence of solely D-lactate dehydrogenase leads to the production of optically pure D-lactate and the introduction of further modifications led to an *E. coli* strain producing up to 0.95 g D-lactate per g glucose (Wendisch *et al.*, 2006). Lactate is also the main fermentative end-product of *C. glutamicum* being exposed to limited oxygen conditions (Inui *et al.*, 2004), and its limited ability to grow at low oxygen concentrations could be an advantage for industrial utilization. Propionate production is performed mostly chemically, but alternative microbiological developments regarding *Propionibacterium acidipropionici* are on the way (Quesada-Chanto *et al.*, 1994; Suwannakham *et al.*, 2006). The production of the C₄-dicarboxylates malate, succinate and fumarate is barely established on the biotechnological scale, but advances were made recently to create an *E. coli* strain, which produced succinate aerobically, yielding 0.85 mol succinate per mol glucose. This strain lacked genes encoding succinate dehydrogenase, acetate kinase and phosphotransacetylase, pyruvate:quinone oxidoreductase, and enzyme II of glucose PTS. Moreover, it had a constitutively active glyoxylate cycle and overexpressed PEP carboxylase (Lin *et al.*, 2005). Alternatively, succinate production could be performed with *C. glutamicum*, which also excretes succinate when incubated anaerobically (Inui *et al.*, 2004). A strain lacking the gene encoding the NAD⁺ dependent lactate dehydrogenase and overexpressing the gene encoding pyruvate carboxylase produced 1.2 mol succinate per mol glucose with a 3-4 fold higher productivity than *E. coli* (Wendisch *et al.*, 2006).

The substrates mentioned so far mostly represent metabolic end products for the respective organism or are accumulated due to their insufficient turnover. The production of pyruvate is additionally challenged due to the central role of this substrate for the cellular metabolism, but the previously mentioned cost effectiveness makes it an interesting target for biotechnological production. Besides the natural pyruvate producing yeast *Torulopsis* (Li *et al.*, 2001), several metabolic modifications turned *E. coli* and *C. glutamicum* into promising pyruvate producers. For *E. coli*, the best result was obtained by limiting the amount of pyruvate consuming reactions by deleting genes encoding subunits of the pyruvate dehydrogenase, pyruvate formate lyase, PEP synthetase, pyruvate:quinone oxidoreductase and NAD⁺ dependent lactate dehydrogenase (Wendisch *et al.*, 2006). For *C. glutamicum*, substantial pyruvate excretion was observed in the strain lacking *aceE* encoding the E1p subunit of the pyruvate dehydrogenase, but no further studies were carried out concerning the improvement of its pyruvate production ability (Blombach *et al.*, 2007a).

In summary, the mentioned biotechnological processes are well established and are able to compete with chemical synthesis but further improvements could result in higher yields and

thus additional cost reduction. Since transport processes are frequent bottlenecks during substrate production, enhanced transport activity by overexpression of the corresponding gene might result in accelerated production, increasing fermenter turnover rates and thus reduce production costs. On the other hand, enhanced import activity might improve the accessibility of a cheap or convenient substrate to the cell and thus accelerate the production process.

1.6 Objectives of this thesis

The aim of this work is to get a maximal possible overview on the systems involved in carboxylate transport by their identification and subsequent characterization in the biotechnologically important organism *C. glutamicum*.

In the first place, the conditions for transport investigation need to be set. For import systems, growth on a certain substrate could indicate its active uptake unless it is facilitated by diffusion. For export systems, the trigger for substrate export has to be identified. Once the conditions are set, the transport mechanism regarding the basic biochemical transporter features as its kinetic properties and substrate specificity can be determined.

The identification of the respective transport system can be performed by various approaches. First, the reverse genetics approach can be addressed by creation of site-directed mutants and their subsequent phenotypic characterization. Second, since the creation of a large transposon mutant collection (Mormann *et al.*, 2006), the forward genetics approach is also accessible. This mutant collection can be screened for mutants with a transporter deficient phenotype and the affected gene can be identified. Third, the transcriptional profile of *C. glutamicum* can be analyzed under conditions, when transport is observed. Differentially expressed transporter encoding genes can be thereupon analyzed for their involvement in carboxylate transport processes.

The knowledge gained in this work could be instantly utilized for the optimization of biotechnological production processes and could be extended on pathogenic strains offering potential new drug targets. Moreover, it would make calculation of substrate fluxes, which largely depend on the exchange with the extracellular surroundings, more accurate thus promoting the overall understanding of the complex metabolic network.

2 Materials and Methods

Trademarks and company names listed in this work are not specially designated but are nevertheless subject to patent-protection.

2.1 Bacterial strains, plasmids and oligonucleotides

2.1.1 Bacterial strains

Bacterial strains used in the study are listed in table 7.1 in the appendix.

2.1.2 Plasmids

Plasmids used in this study are listed in table 7.2 in the appendix.

2.1.3 Oligonucleotides

The oligonucleotides used in this work are listed in table 7.3 in the appendix.

2.2 Cultivation of bacteria

2.2.1 Cultivation of *E. coli*

E. coli strains were routinely grown at 37°C in LB (Luria-Bertani) medium (table 2.1) or on LB medium solidified by addition of 1.5% w/v Bacto-Agar (Difco, Detroit, USA) and supplemented with 50 µg/ml carbenicillin or 25 µg/ml kanamycin. The plates were additionally supplemented with 100 mM IPTG (isopropyl-D-thiogalactopyranoside) and 80 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) if selection of plasmids with interrupted *lacZ* sequence was performed. The growth of the bacterial cultures was determined by the optical density (OD₆₀₀). An OD₆₀₀ of 1 corresponds to a bacterial suspension of approx. 10⁹ cells per ml.

2.2.2 Cultivation of *C. glutamicum*

C. glutamicum strains were grown at 30°C in BHI medium (Brain Heart Infusion, Difco, Detroit, USA) or on BHI solidified by addition of 1.5% w/v Bacto-Agar with 25 µg/ml kanamycin, when appropriate. To obtain reproducible results, the inoculation procedure has been standardized. First, 5 ml BHI medium were inoculated with *C. glutamicum*. After 6-8 h at 30°C, 5 ml MM1 medium (table 2.1, Kase and Nakayama, 1972) supplemented with 200 mM glucose was inoculated with this culture and incubated over night at 30°C, constantly shaking. In the morning, a new MM1 medium supplemented with a defined carbon source was inoculated with the over-night culture to an OD₆₀₀ of 1.0 (or appropriate). For oxygen limitation, cells were grown in MM1 medium up to mid-exponential phase (4-5 h) and concentrated to an OD₆₀₀ of approx. 25 by centrifugation (4.000 rpm, 5 min, 30°C) and

concomitant resuspension in fresh MM1 medium. The culture was incubated in sealed reaction tubes at 30°C.

Table 2.1 Media used in the study:

Medium	Ingredients (per l)
LB	10 g Tryptone, 5 g yeast extract, 10 g NaCl
BHI	37 g/l Brain Heart Infusion
MM1	5 g (NH ₄) ₂ SO ₄ , 5g urea, 2 g KH ₂ PO ₄ , 2 g K ₂ HPO ₄ , 1.46 g NaCl, pH (KOH) = 7.0; after autoclavation addition of sterile filtered 0.25 g MgSO ₄ , 0.01 g CaCl ₂ , 200 µg biotin, 10 mg FeSO ₄ , 10 mg MnSO ₄ , 1 mg ZnSO ₄ , 0.2 mg CuSO ₄ , 0.02 mg NiCl ₂ , 0.09 mg H ₃ BO ₃ , 0.06 mg CoCl ₂ , 0.009 mg NaMoO ₄

2.3 Molecular biological approaches

2.3.1 Genetic manipulation of bacteria

2.3.1.1 Preparation of competent *E. coli* cells

To prepare competent *E. coli* cells, 5 ml LB were inoculated and incubated at 37°C for 8 h. 1 ml of this culture was used to inoculate 250 ml SOB medium in a 2 l Erlenmeyer-flask. Cells were grown over night at 25°C, constantly shaking. The culture was chilled on ice for 10 min after having reached an OD₆₀₀ of approximately 0.6. Cells were harvested by centrifugation (2.500 rpm, 5 min, 4°C) and resuspended in 50 ml ice-cold TB buffer. After 10 min of incubation on ice, cells were centrifuged again and resuspended in 20 ml TB buffer. 1.4 ml DMSO were added drop wise to the cells, which were then incubated on ice for 10 min. The cell suspension was aliquoted to 100 µl in pre-cooled Eppendorff tubes, immediately frozen in liquid nitrogen and stored at –80°C.

SOB medium: 20 g/l Tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, and 2.5 mM KCl; after heat sterilization, 5 ml sterile 2 M MgCl₂ were added.

TB buffer: 10 mM Pipes, 15 mM CaCl₂, 250 mM KCl, pH (KOH) = 6.7. After adjustment of pH, 6.95 g/l MnCl₂ were added. Sterilization by filtration.

2.3.1.2 Preparation of competent *E. coli* cells (TSS-method)

To quickly prepare competent *E. coli* cells for direct transformation, 5 ml LB were inoculated and incubated at 37°C for 3-4 h to an OD₆₀₀ of 0.3-0.5. 1.5 ml of this culture were collected by centrifugation (10.000 rpm, 1 min, 4°C) and resuspended in 400 µl ice cold TSS solution. 100 µl of this cell solution was directly used for transformation.

TSS buffer: 10 g/l Pepton, 10 g/l NaCl, 5 g/l yeast extract, 5% DMSO, and 20 mM MgSO₄. Sterilization by filtration.

2.3.1.3 Transformation of competent *E. coli* cells

For transformation of competent *E. coli*, 2 µl plasmid or 5 µl ligation mix (conf. 2.3.2.6) were added to an aliquot of *E. coli* cell solution and incubated on ice for at least 30 minutes. Afterwards, the cells were heat shocked for 45 seconds at 42°C in a shaking incubator. After a subsequent cooling on ice for 5 minutes, 600 µl SOC medium were added and the cells were incubated at least 1 h at 37°C in a shaking incubator. Cells were harvested by centrifugation (3.000 rpm, 1 min, RT), plated on LB plates with the appropriate antibiotic and cultivated at 37°C for 16 h.

SOC medium: 3.6 g glucose were added to 1 l SOB medium (2.3.1.1) and sterilized by filtration.

2.3.1.4 Preparation of competent *C. glutamicum* cells

To obtain competent *C. glutamicum* cells, 20 ml LB medium with 2% glucose were inoculated and cultivated at 30°C for 8 h under constant shaking. Subsequently, this culture was used to inoculate 200 ml LB medium with growth inhibitors to an OD₆₀₀ of 0.2-0.3. These cells were cultured over night at 25°C, chilled on ice for 10 minutes and harvested by centrifugation (4.000 rpm, 5 min, 4°C). The cells were washed five times in ice-cold 10% glycerol. After the final washing, the cell pellet was resuspended in 1 ml ice-cold 10% glycerol. Aliquots of 50 µl were transferred into pre-cooled reaction tubes, immediately frozen in liquid nitrogen and stored at -80°C.

LB medium with growth inhibitors: 10 g/l Tryptone, 5 g/l yeast extract, 5 g/l NaCl, 4 g/l isonicotinic acid hydrazide, 25 g/l glycine, 0.1% (v/v) Tween 80, sterilization by filtration.

2.3.1.5 Transformation of competent *C. glutamicum* cells

For transformation, a 50 µl aliquot of competent *C. glutamicum* cells was thawed on ice and transferred to a pre-cooled electroporation cuvette (Biorad, München, Germany). 2 µl plasmid DNA was added and electroporation was performed with a Gene-Pulser (Biorad, München, Germany) at 2.5 kV, 600 Ω, and 25 µF. After that, 1 ml BHIS medium was added and the cells were cultivated for at least 2 h at 30°C, constantly shaking. Cells were harvested by centrifugation (3.000 rpm, 3 min, 25°C), resuspended in 100 µl BHIS medium and plated on a BHI plate containing the appropriate antibiotic.

BHIS medium: 37 g/l Brain Heart Infusion, 91 g/l sorbitol; sterilization by filtration.

2.3.1.6 Generation of insertion strains of *C. glutamicum*

To introduce an insertion into the chromosome of *C. glutamicum*, a part of the target sequence (usually 500-1000 bp) was amplified by PCR (2.3.2.4) and ligated into the insertion vector pDrive by A-T-cloning (2.3.2.6). The resulting plasmid was used for electro-transformation of competent *C. glutamicum* cells. After an initial incubation on BHI agar plates containing 15 µg/ml kanamycin for 48 h at 30°C, clones were transferred on BHI agar plates with 25 µg/ml kanamycin and incubated for 24 h at 30°C. The successful insertion of the vector was proven by PCR with several primer combinations as indicated below.

<u>Primer combination:</u>	<u>Expected result:</u>
1) Amplified target sequence (Forward)/ Amplified target sequence (Reverse)/	PCR product
2) Amplified target sequence (Forward)/ M13 Universal -21	PCR product, if previously amplified fragment was ligated 3`-5` into pDrive
3) Amplified target sequence (Forward)/ M13 reverse	PCR product, if previously amplified fragment was ligated 5`-3` into pDrive
4) M13 Universal -21/ M13 reverse	no PCR product

2.3.1.7 Generation of deletion strains of *C. glutamicum*

To introduce a chromosomal deletion in the *C. glutamicum* genome, a method similar to the protocols described by Schäfer *et al.* (1994) and Niebisch and Bott (2001) was used. The flanking genomic sequences of the target gene were cloned into the plasmid pK18*mobsacB*. *C. glutamicum* was transformed with the resulting plasmid. Cells carrying the plasmid were selected by their resistance to kanamycin and their sensitivity to sucrose. A single colony carrying the plasmid was used to inoculate 3 ml LB medium containing 2% (w/v) glucose and 25 µg/ml kanamycin. After over-night growth at 30°C, cells were harvested by centrifugation (14.000 rpm, 30 sec, RT) and washed two times with 3 ml CgXIIoNoP medium. Finally, the cells were resuspended in 3 ml CgXIIoNoP medium and cultivated for 6 h at 30°C in the absence of kanamycin. After that, the culture was diluted in LB 1:1.000 and 1:10.000, respectively. 150 µl of this cell suspension were plated on BHI plates containing 10% sucrose. Colonies that were resistant to sucrose and sensitive to kanamycin were analyzed by PCR to identify clones carrying the desired deletion.

CgXIIoNoP: 42 g/l MOPS, pH (NaOH) = 7.0, after heat sterilization addition of 1 mM MgSO₄, 0.1 mM CaCl₂, 0.2 mg/l biotin, 10 mg FeSO₄, 10 mg MnSO₄, 1 mg ZnSO₄, 0.2 mg CuSO₄, 0.02 mg NiCl₂, 0.09 mg H₃BO₃, 0.06 mg CoCl₂, 0.009 mg NaMoO₄, 0.5% glucose, 1.8 mg/l protocatechuic acid (pre-solved in NaOH).

2.3.2 DNA techniques

2.3.2.1 Isolation of genomic DNA

To obtain genomic DNA from bacteria, 3 ml of an over-night culture were spinned down (15.300 rpm, 1 min, 4°C) and resuspended in 200 µl H₂O. After addition of 200 µl Roti-Phenol, the sample was incubated at 65°C for at least 10 min and regularly vortexed. To remove the phenol, 200 µl chloroform were added after a short cool down on ice. The sample was centrifuged (15.300 rpm, 10 min, 4°C) and the aqueous phase was washed once more with 200 µl chloroform. The DNA was precipitated for at least 30 min with 150 µl acetate/acetic acid solution and 1 ml 98% ethanol. Afterwards, the DNA was isolated by centrifugation (15.300 rpm, 15 min, 4°C), washed with 200 µl 70% ethanol, dried and resuspended in 50 µl H₂O.

Alternatively, the DNA was isolated without the application of phenol. Therefore, the isolation of genomic DNA from *C. glutamicum* was performed as described by Eikmanns *et al.* (1994). *C. glutamicum* cells were cultivated overnight in 5 ml BHI medium, harvested by centrifugation (14.000 rpm, 5 min, RT) and washed twice in TE buffer. Thereupon, the cells were incubated for 3 h at 37°C in 1 ml TE buffer supplemented with 15 mg lysozyme (Sigma, Deisenhofen, Germany). 200 µl 10% SDS was added and the sample was gently mixed. After 2 min, 3 ml lysis buffer and 125 µl proteinase K (Roche Diagnostics, Mannheim, Germany) were added and incubated for 16 h at 37°C. To remove proteins, 2 ml of a saturated NaCl solution was added and the suspension was gently mixed. After centrifugation (4.000 rpm, 30 min, RT), the supernatant was transferred to a new tube and ice-cold ethanol was added to a final volume of 15 ml in order to precipitate the DNA. Genomic DNA was washed with 70% ethanol, dried, and resuspended in water.

TE: 10 mM Tris, 1 mM EDTA, pH (HCl) = 7.5

Lysis-Buffer: 10 mM Tris, 400 mM NaCl, 2 mM EDTA, pH (HCl) = 8.2

acetate/acetic acid solution: 60 ml 5 M K⁺-acetate, 11.5 ml acetic acid, 28.5 ml H₂O

2.3.2.2 Isolation of plasmid DNA from *E. coli*

For plasmid isolation, *E. coli* cells were grown in 3 ml LB medium with the appropriate antibiotic at 37°C for 16 h. For plasmid isolation, the NucleoSpin Plasmid DNA Purification kit (Macherey-Nagel, Düren, Germany) was used as recommended by the supplier.

2.3.2.3 Gel electrophoresis and extraction of DNA from agarose gels

Gel electrophoresis of DNA was performed in 0.8 – 2% agarose gels in TAE buffer as described by Sambrook *et al.* (1989). Samples were mixed with 6x Loading Dye (MBI Fermentas, St. Leon-Roth, Germany). After electrophoresis, DNA was stained with ethidium bromide. For detection of stained DNA, the Image Master VDS system (Amersham Biosciences, Freiburg, Germany) was used. DNA was isolated from agarose gels using the NucleoSpion Extract kit (Macherey-Nagel, Düren, Germany) as recommended by the supplier and eluted from columns with 15 µl H₂O.

1x TAE buffer: 40 mM Tris, 0.5 mM EDTA, pH (acetic acid) = 7.5

6x Loading Dye: 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA, 10 mM Tris HCl (pH 7.6)

2.3.2.4 PCR

The selective *in vitro* amplification of specific DNA fragments was performed by polymerase chain reaction (PCR, Mullis *et al.*, 1986) using the Taq PCR Master Mix (Eppendorff, Hamburg, Germany) as recommended by the supplier. Primers were supplied by Operon (Cologne, Germany). For a total reaction volume of 10 µl, 10 pmol of each primer flanking the amplified DNA region and 1 µl of the template DNA solution were added. Alternatively, a cell colony was resuspended in 100 µl H₂O, boiled for 15 min at 95°C, the cell debris was removed by centrifugation (14.000 rpm, 1 min, RT) and 1 µl from the supernatant was used for the PCR. The PCR was performed with a Mastercycler personal or Mastercycler gradient (Eppendorff, Hamburg, Germany). Either a standard or a touch-down PCR program was used. The melting temperature was calculated by the IDT SciTools OligoAnalyzer 3.0 (<http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>) and the annealing temperature of the PCR was adjusted to approx. 5°C below the melting temperature of the primer. The elongation time was set to 1 min per amplified kb with additional 30 sec. The primers used are listed in table 7.3 in the appendix.

Standard PCR program

1) 94°C	4'	
2) 94°C	30"	} repeat 30x
3) 45-65°C	30"	
4) 72°C	90"-210"	
5) 72°C	7'	
6) 4°C	∞	

Touch-down PCR program

1) 94°C	4'		14) 94°C	30"	} repeat 2x
2) 94°C	30"	} repeat 2x	15) 50°C	30"	
3) 59°C	30"		16) 72°C	90"	
4) 72°C	90"	} repeat 2x	17) 94°C	30"	} repeat 2x
5) 94°C	30"		18) 48°C	30"	
6) 57°C	30"		19) 72°C	90"	
7) 72°C	90"	} repeat 2x	20) 94°C	30"	} repeat 18x
8) 94°C	30"		21) 50-65°C	30"	
9) 54°C	30"		22) 72°C	90"	
10) 72°C	90"	} repeat 2x	23) 72°C	7'	
11) 94°C	30"		24) 4°C	∞	
12) 52°C	30"				
13) 72°C	90"				

2.3.2.5 Restriction of DNA

For restriction of DNA, restriction enzymes were used as recommended by the supplier (NEB, Frankfurt/Main, Germany). When religation of plasmids was not desired, 1 µl shrimp alkaline phosphatase (NEB, Frankfurt/Main, Germany) was added to dephosphorylate 5' ends. After restriction, DNA was purified by gel electrophoresis and subsequent elution with the NucleoSpin Extract kit (Macherey-Nagel, Düren, Germany) following the supplier's protocol.

2.3.2.6 Ligation of DNA

For the ligation of DNA fragments with restricted vectors, the T4 DNA Ligase (MBI Fermentas, St. Leon-Roth, Germany) was used as recommended by the supplier. For direct ligation of PCR products in the pDrive vector by A-T-cloning, the QIAGEN PCR Cloning kit (Qiagen, Hilden, Germany) was used following the supplier's protocol. After ligation, 5 µl of the reaction mix was used to transform competent *E. coli* cells (conf. 2.3.1.3).

2.3.2.7 Sequencing of DNA

DNA sequence analyses were carried out by the bioanalytics service unit at the Center for Molecular Medicine Cologne (ZMMK) or by GATC Biotech AG (Konstanz, Germany). 500 ng of plasmid DNA were provided for this purpose.

2.3.2.8 Determination of the Tn integration site

C. glutamicum Tn mutants were created by transformation with the pAT6100 vector carrying the is6100 element (Mormann *et al.*, 2006). The linearized vector was integrated into the *C. glutamicum* chromosome (Fig. 2.1) due to the action of the is6100 encoded transposase.

In order to localize the Tn element in the *C. glutamicum* genome, the DNA was extracted (2.3.2.1), digested with *EcoRI* or *XbaI* (2.3.2.5), ligated (2.3.2.6) and the resulting plasmid was used for *E. coli* transformation (2.3.1.3). The plasmid transmits kanamycin resistance and only the OriV containing plasmids can be replicated in the new host. Sequencing was performed with s6100x primer after *XbaI* digestion or with s6100e primer after *EcoRI* digestion.

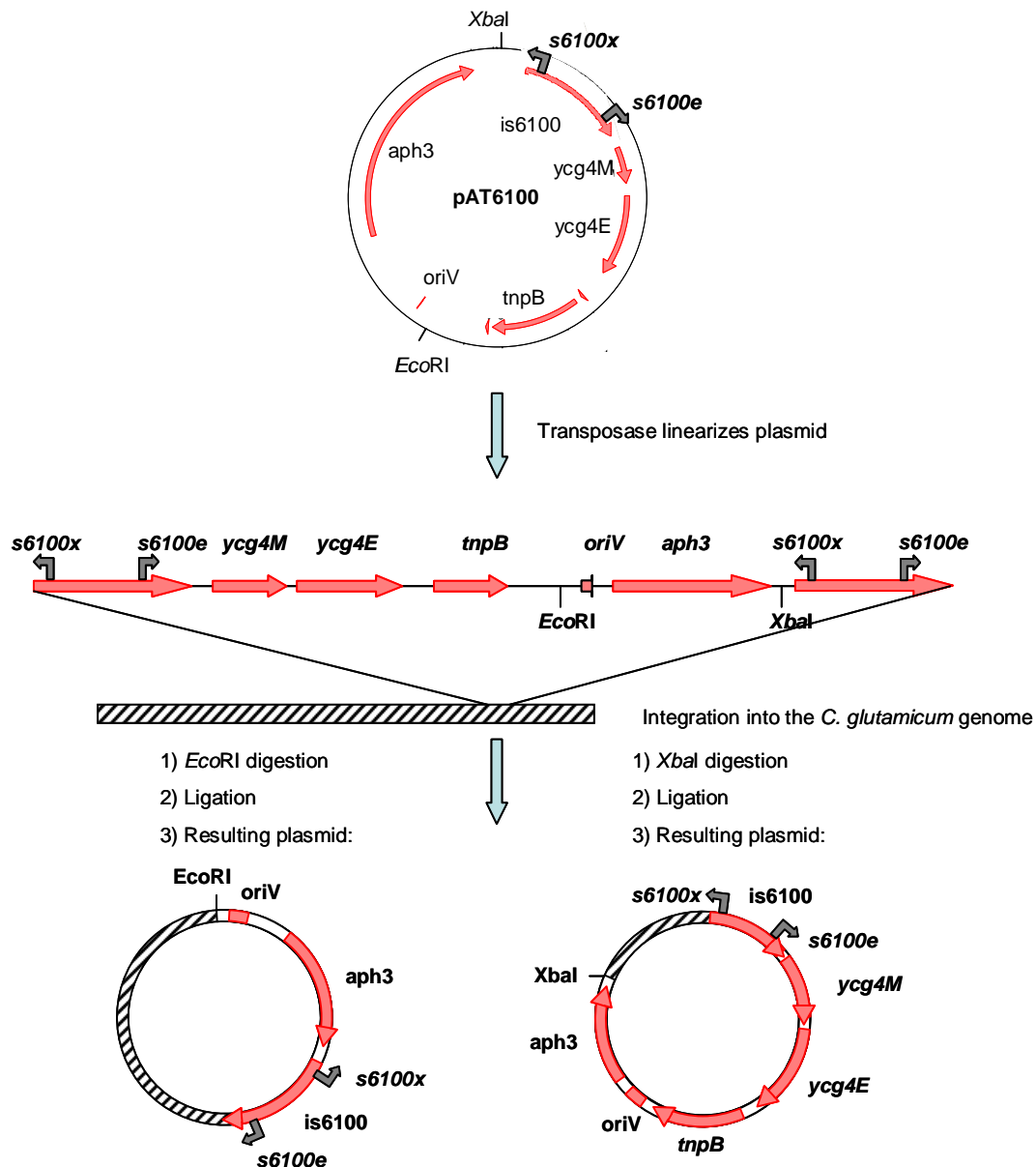


Fig. 2.1 Schematic overview on the integration of *is6100* and the plasmid rescue technique. Flexed arrows indicate the sequencing primer binding site. Striped elements indicate *C. glutamicum* genome.

2.3.3 RNA techniques

2.3.3.1 Isolation of total RNA from *C. glutamicum*

For the isolation of total RNA from *C. glutamicum*, cells were exposed to different conditions. 2 ml of the culture were collected by centrifugation (14.000 rpm, 30 sec, 4°C) and the resulting pellet was immediately frozen in liquid nitrogen and stored at -80°C. For the preparation of RNA, the cell pellet was thawed at room temperature and subsequently diluted in 350 µl RA1 buffer (NucleoSpin RNA II Kit, Macherey-Nagel, Düren, Germany) supplemented with 1% (v/v) β-mercaptoethanol. The resulting suspension was transferred into cryo tubes containing 300 mg glass beads (diameter 0.2-0.3 mm). Cell disruption was performed by 3 cycles of vigorous shaking in the FastPrep FP120 instrument (Q-BIOgene, Heidelberg, Germany) for 30 sec at 6.5 m/s. The cell debris and glass beads were subsequently removed by centrifugation (14.000 rpm, 2 min, 4°C) and the supernatant was mixed with 350 µl 70% ethanol. In the following, total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) as recommended by the supplier. The integrity of isolated total RNA was analyzed by gel electrophoresis. For this purpose, 3 µl of purified total RNA was mixed with 10 µl RNA loading buffer and denatured 10 min at 70°C. Subsequently, the samples were kept on ice for 5 min. As quality control, gel electrophoresis and detection of RNA was performed in accordance to the analysis of DNA described in section 2.3.2.3.

For subsequent RT-PCR analyses, total RNA was additionally treated with RQ1 RNase-free DNase (Promega, Mannheim, Germany) as recommended by the supplier.

RNA loading buffer: 250 µl formamide, 83 µl 37% formaldehyde, 50 µl 10x MOPS buffer, 50 µl glycerol, 0.01% bromphenol blue, 0.01% xylene cyanole, 1 µl 10 g/l ethidiumbromide, 120 µl RNase-free H₂O

MOPS buffer: 200 mM MOPS, 50 mM Na⁺ acetate, 10 mM EDTA, pH = 7.0

2.3.3.2 RT-PCR

The identification of specific mRNA transcripts in total RNA samples was performed by means of RT-PCR. For this purpose, the Revert Aid H Minus First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany) was used as recommended by the supplier. Total RNA treated with RQ1-DNase served as template. First, the mixture containing 3 µg total RNA, 1 µl random hexamer primer and RNase free H₂O ad 12 µl was incubated 5 min at 70°C and subsequently chilled on ice. The reaction buffer, RiboLock Ribonuclease Inhibitor and dNTPs were added and the mixture was incubated for 5 min at 37°C. The reverse transcriptase was added and the sample was incubated at 25°C for 10 min and finally at 42°C for 1 h. The reaction was stopped by heating at 70°C for 10 min. After chilling

on ice, 1 µl of this reaction was directly used for subsequent standard PCR reaction (2.3.2.4).

2.3.3.3 DNA-Microarray analysis

Cells were collected by rapid centrifugation (14000 rpm, 10 sec, 4°C) and were immediately frozen in liquid N₂ after removal of the supernatant. RNA isolation was performed with the innuPREP RNA Mini Kit (Analytic Jena, Jena, Germany). Therefore, the frozen pellet was resuspended in 750 µl Lysis Solution RL and mechanically disrupted (6.5 m/s, 20 sec) (Hybaid Ribolyser, Heidelberg, Germany). RNA was bound to the Spin Filter R according to the manufacturer's instructions. DNase digestion was performed on the filter with 10 µl DNase (Roche Diagnostics, Mannheim, Germany) for 80 min at 37°C. RNA was washed and eluted in 80 µl H₂O.

4 µg RNA were used for cDNA synthesis. Therefore, RNA was supplemented with AmC₆ random hexanucleotides, denatured at 70°C for 10 min and subsequently cooled down on ice (5 min) in order to anneal the hexanucleotide primer to the RNA. Buffer, dNTPs and RiboLock were added according to the supplier's instructions (Fermentas, St. Leon-Roth, Germany). 300 U Bioskript reverse transcriptase (Bioline, Luckenwalde, Germany) were added and the samples were incubated on ice for further 5 min. The samples were placed in a thermomixer at 10°C and the temperature was raised to 42°C, which was hold for 90 min. In order to remove remaining RNA from the cDNA samples, the synthesized cDNA was supplemented with 10 µl 50 mM EDTA and 10 µl 1 M NaOH. The RNA was hydrolyzed at 70°C for 10 min and the pH was adjusted by the addition of 10 µl 1 M HCl. cDNA was purified with the aid of the ChargeSwitch Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

In order to label the cDNA with a fluorescent dye for the microarray assay, the dyes Cy3 and Cy5 (GE Healthcare, München, Germany) were used. The dyes were dissolved in 16.5 µl DMSO and 2 µl were vacuum dried for each cDNA sample. The dry dye pellet was dissolved in the purified cDNA containing solution and cDNA labelling was performed for 60 min at RT in darkness. The free reactive sites of the Cy3/Cy5 dye were saturated by the addition of 4.5 µl 4 M hydroxylamine and the unbound dye was removed in a sephadex column (Princeton Separations, Berlin, Germany).

Cg3kOligo microarrays were prepared and hybridized with the labelled cDNA in a HS4800 hybridization station by the transcriptomics facility of the University of Bielefeld, Germany.

2.4 Biochemical approaches

2.4.1 Protein isolation

Cells were grown in BHI or MM1 medium, harvested by centrifugation (4.000 rpm, 5 min, 4°C), washed with washing buffer and resuspended in lysis buffer. Cells were disrupted

mechanically with glass beads in Bio101 Savant FastPrep FP120 (4 x 30 sec at 6.5 m/s) and cell debris was removed by centrifugation (14.000 rpm, 10 min, 4°C). In order to separate membrane proteins from total cell protein, the membrane fraction was separated by centrifugation (Optima TLX Ultracentrifuge, 228.000 g, 30 min, 4°C) and resuspended in 50 µl lysis buffer.

Washing buffer: 25 mM KH₂PO₄, 100 mM NaCl, pH 7.5

Lysis buffer: 25 mM KH₂PO₄, 100 mM NaCl, 1 mM EDTA, 5 µg/ml DNase, 1 tablet/100 ml Complete (Roche Diagnostics, Mannheim, Germany), pH 7.5

2.4.2 Determination of protein concentration

The protein content of cell extract was measured as described by Bradford (1976). For this purpose, 5 to 10 µl of cell extract were diluted in 200 µl H₂O and supplemented with 800 µl 1:5 diluted Roti-Nanoquant reagent (Carl Roth GmbH, Karlsruhe, Germany). The absorbance of the samples was measured at 590 nm and 450 nm and the ratio A_{590}/A_{450} was calculated. The concentration of the protein solution could be determined by the use of a BSA (NEB, Frankfurt/Main, Germany) standard curve.

2.4.3 Enzymatic assays

2.4.3.1 Pyruvate Dehydrogenase activity assay

Cells were grown for 6 h to late exponential phase in BHI (Fiur, 2004). Cell protein was isolated by mechanical cell disruption in Bio101 Savant FastPrep FP120 (3 x 30 sec at 6.5 m/s) in homogenization buffer. Cell debris was removed by centrifugation (14.000 rpm, 15 min, 4°C). The enzyme assay was carried out immediately. Therefore, 50 µl of supernatant was supplied with 950 µl reaction buffer. The reaction was started by the addition of 25 µl 8 mM CoA solution. NADH formation was followed photometrically at 340 nm and the PDH activity calculated accordingly. The millimolar extinction coefficient of NADH is 6.22 mmol⁻¹ cm⁻¹. i.e. 1 mmol NADH ml⁻¹ has an extinction at 340 nm of 6.22.

Homogenization buffer: 100 mM Tris, 10 mM MgCl₂, 3 mM L-cysteine, pH (HCl) = 7.2

Reaction buffer: 100 mM Tris, 1 mM thiaminpyrophosphate, 5 mM pyruvate, 3 mM L-cysteine, 10 mM MgCl₂, 2 mM NAD⁺, pH (HCl) = 7.2

2.4.3.2 Lactate assay

A convenient volume of supernatant (usually 5 µl) was added to 100 µl 2x reaction buffer and H₂O was added to a final volume of 200 µl. NAD⁺ was measured in the Wallac

Multilabel Counter at 340 nm and 1.375 U L-LDH (Roche Diagnostics, Mannheim, Germany) were used to start the reaction. NADH formation was measured at 340 nm after 1 h incubation at 30°C. Lactate concentration was calculated according to a standard curve.

2x reaction buffer: 600 mM glycine, 800 mM hydrazine, 4.8 mM NAD⁺, pH (NaOH) = 9.0

2.4.3.3 Pyruvate assay

A convenient volume of supernatant (usually 20 µl) was added to 100 µl 100 mM Tris-HCl (pH = 7.4), 58 µl H₂O and 20 µl 1.4 mM NADH. NADH was measured in the Wallac Multilabel Counter at 340 nm and 1.375 U L-LDH (Roche Diagnostics, Mannheim, Germany) were used to start the reaction. NADH oxidation was measured at 340 nm after 1 h incubation at 30°C. Pyruvate concentration was calculated according to a standard curve.

2.5 Analytic methods

2.5.1 Sample preparation

To determine extracellular concentrations of substrates of interest, cells were grown as described previously (conf. 2.2.2). 1 ml of the culture was spun down (14.000 rpm, 5 min, 4°C). For HPLC, salts were directly removed by prolonged centrifugation (14.000 rpm, 20 min, 4°C) and the sample was directly applied on the HPLC column. For GC, 100 µl of the supernatant were supplemented with 100 µg ribitol as internal standard and vacuum dried at 60°C. The residue was dissolved in 65 µl pyridine and incubated for 90 minutes at 30°C. 35 µl MSTFA (n-methyl-n-trimethylsilyl-trifluoroacetamid) were added and the sample was incubated for 60 minutes at 65°C. The resulting solution was analyzed by gas chromatography.

To determine intracellular substrate concentrations, cells were harvested by silicone oil centrifugation. Therefore, microcentrifuge tubes were loaded with 30 µl 20% perchloric acid and 70 µl silicone oil (d=1.04 g/cm³) and centrifuged briefly. 200 µl of the culture was placed on top of the silicone oil. For each sample, three microcentrifuge tubes were loaded. Cells were passed through the oil layer by centrifugation (45 sec, RT) in Microfuge E (Beckman Instruments GmbH, München, Germany). The cell pellet was separated from the silicone oil layer by cutting off the lower part of the microcentrifuge tube. The pellet was transferred into a new tube by short centrifugation. The samples were replenished with 135 µl H₂O and 100 µl silicone oil (d=1.06 g/cm³). After 10 min of sonification, the solution was neutralized with 75 µl of a solution of 5 M KOH and 1 M triethanolamine and incubated for 30 min at 4°C. Salts and cell debris were removed by centrifugation (14.000 rpm, 20 min, 4°C). The sample was either directly applied on a HPLC column or 100 µl of the supernatant were supplemented with 100 µg ribitol and prepared for GC analysis as described above.

2.5.2 Gas chromatography (GC)

For the quantitative analysis of organic acids, the pyridine/MSTFA solutions obtained after sample preparation was analyzed by gas chromatography using the TraceGC system (Thermo Finnigan, Woburn, USA) and the FS-Supreme 5 column (CS-Chromatographie, Langerwehe, Germany). 0.5 µl of the samples were injected (split ratio 1:25). The injector temperature was set at 50°C. Separation of the ingredients was performed with nitrogen carrier gas (flow rate: 1 ml/min) using the following time program: 2 min at 60°C, temperature gradient of 30°C/min up to 280°C, holding 280°C for 2 min, temperature gradient of 30°C/min up to 320°C, holding 320°C for 4 min. At the end of the run, initial conditions were restored. Column effluents were monitored by flame ionization detection (FID) at 300°C. The air flow of the detector was set at 350 ml/min and the H₂ flow at 35 ml/min. Substrate concentration within a sample was calculated according to a standard curve.

2.5.3 High-pressure liquid chromatography (HPLC)

For the quantitative analysis of organic acids, samples were prepared as described in 2.5.1. The concentration of the carboxylic acids was determined using the LaChrom Elite HPLC system (VWR, Darmstadt, Germany). Column effluents were monitored with a UV-detector (VWR, Darmstadt, Germany) at a detection wavelength of 210 nm and an RI-detector (VWR, Darmstadt, Germany). Solutes were separated by means of a VA300/7.8 Nucleogel Sugar 810H sulfonated polystyrene/divinylbenzene resin containing column (Macherey-Nagel, Düren, Germany) at 40°C with 0.01 M H₂SO₄ as mobile phase with a flow rate of 0.5 ml/min.

2.5.4 Thin layer chromatography (TLC)

Radioactively labelled organic acids were analyzed for their integrity using thin layer chromatography (TLC) on silica gel-coated plates (60 F₂₅₄, 0.2 mm thickness, Merck, Darmstadt, Germany) with n-butanole/acetic acid/H₂O [4:1:1, (v/v/v)] as mobile phase. The plates were dried and exposed overnight to storage Phosphor Imaging Plates (BAS-IP MP 2025, Fujifilm, Düsseldorf, Germany). Substrate spots were detected by the use of a phosphorimager (Fujifilm BAS-1800, Fujifilm, Düsseldorf, Germany).

2.6 Monitoring carboxylic acid uptake

To monitor uptake of carboxylic acids by *C. glutamicum*, cells were grown in MM1 supplied with 50 mM of the carboxylic acid to be transported. In case of propionate uptake, the cells were pre-cultivated on a mixture of 50 mM acetate and 50 mM propionate. Medium for *imctC* was supplied with 50 mM glucose. Medium for *C. glutamicum* pVWEx1-*dccT* was supplemented with 1 mM IPTG and for *C. glutamicum* pVWEx1-*dctA* with 25 µM IPTG. After

reaching the mid-exponential growth phase, the cells were washed three times with MES/Tris buffer and incubated on ice until the actual measurement. Prior to the addition of the transported substrate, 1.5 ml cells were incubated for 3 min at 30°C with 10 mM glucose. ^{14}C labelled substrate (MP-Biochemicals, Illkirch, France) was added with the unlabelled one in the desired concentration and samples were taken. In the case of pyruvate, L-malate, or fumarate uptake, samples were taken each 30 sec for 2 min, in the case of acetate, propionate, lactate and succinate each 5 sec for 20 sec. Cells were collected on glass fiber filters (Millipore, Eschborn, Germany or Schleicher-Schuell, Dassel, Germany) on a manifold filtration device and washed twice with 2.5 ml 0.1 M LiCl. After resuspension of cells in scintillation fluid (Rotiszint, Roth Germany) radioactivity of the sample was counted in a scintillation counter (Beckmann, Krefeld, Germany). Uptake rates were calculated according to the formula:

$$\text{Uptake rate [nmol min}^{-1} \text{ (mg cdm)}^{-1}] = (\text{cpm}_{\text{int}} \times \text{substrate in sample } [\mu\text{Mol}]) / (\text{cpm}_{\text{tot}} \times \text{cdm})$$

cpm_{int} : intracellular counts per minute

cpm_{tot} : total counts per minute

cdm : $\text{OD}_{600} \times \text{sample volume [ml]} \times 0.36 [\text{mg ml}^{-1}]$

MES/Tris buffer: 50 mM MES, 50 mM Tris, 10 mM NaCl, 10 mM KCl, pH (HCl) = 8.0

2.7 Bioinformatic approaches

2.7.1 Primer generation

Primer pairs for certain DNA regions were created with the aid of the SECentral software package. When appropriate, primers were designed manually, taking into consideration melting temperature, primer dimer formation, secondary structures, false priming, abundant repetition of bases and the abundance of guanine and/or cytosine within the primer sequence with the aid of IDT SciTools OligoAnalyzer 3.0 (<http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>).

2.7.2 Sequence identification and alignment

Amplified (2.3.2.4) and plasmid-cloned (2.3.2.6) DNA regions were sequenced (2.3.2.7) before creation of deletion mutants. Due to the fragment's length, sequencing was performed from both sides and the sequences were aligned with NCBI BLAST for two sequences (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>), in order to obtain one complete sequence of the amplified region. The resulting sequences were compared with *Corynebacterium glutamicum* ATCC 13032 (Kyowa) genome with the aid of GIB BLAST2 search (http://gib.genes.nig.ac.jp/single/index.php?spid=Cglu_ATCC13032).

3 Results

The experiments presented in the following were aimed at the identification of carboxylate transporters in the industrially relevant organism *C. glutamicum*, the validation as such of proposed transporter candidates and their biochemical characterization. First, the utilization and import of several carboxylates will be considered. Hereafter, their production and export will be described.

3.1 Growth of *Corynebacterium* on carboxylates

C. glutamicum is a widely distributed soil organism. As such, it is expected to grow on a variety of different carbon sources. Here, a systematic analysis of the ability of the laboratory *C. glutamicum* strain to utilize and thus potentially to import carboxylates was performed. Therefore, growth experiments were carried out in minimal medium containing 50 mM carboxylates as sole carbon and energy source. The chosen substrates were either fermentative end-products frequently occurring in soil or in bioreactors, the central metabolite pyruvate, and intermediates of the TCA. Due to the enzyme set of *C. glutamicum*, the utilization of the chosen substrates upon entering the cell was expected.

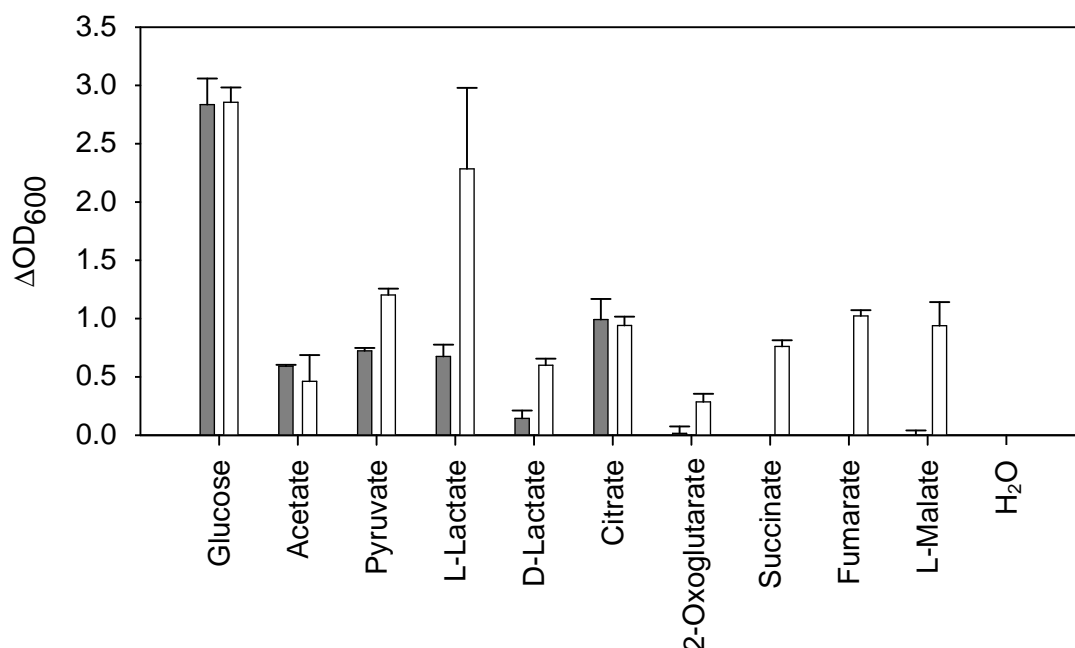


Fig. 3.1 Growth of *C. glutamicum* ATCC 13032 (filled bars) and *C. efficiens* DSM 44549 (open bars) on different carbon sources. Growth was monitored in microtiter plates upon the addition of 50 mM substrate and the change in OD₆₀₀ after 56 h is depicted. n=3.

C. glutamicum showed growth only on a limited number of organic acids (Fig. 3.1). As expected, it grew on the monocarboxylates acetate, pyruvate, lactate and the tricarboxylate citrate. However, no growth was observed on other intermediates of the TCA. In order to validate this result, the growth experiment was performed with *C. efficiens*, which is also a

widely distributed soil organism and was thus expected to grow on carboxylates. As expected and in contrast to *C. glutamicum*, this strain grew on all applied substrates (Fig. 3.1).

The negative result concerning growth of *C. glutamicum* on dicarboxylates indicates either (I) that it is not able to utilize intermediates of the TCA for growth or (II) that their import was only sufficient for metabolic maintenance but not for biomass production or (III) that it is lacking import systems for these substrates or (IV) that the conditions set in this experiment did not match the conditions required for dicarboxylate uptake. The inability to utilize TCA intermediates is unlikely, since the conversion of all TCA intermediates is experimentally proven (Eikmanns, 2005). Thus, either the absence of a dicarboxylate importer or its presence only under special conditions has to be considered. The striking differences between *C. glutamicum* and *C. efficiens* regarding their substrate spectrum can thus be related either to their transporter equipment or to the regulation of carboxylate metabolism. On the other hand, the growth ability of *C. glutamicum* on acetate, both isoforms of lactate, pyruvate and citrate supported the idea that import systems for these substrates are present in this strain. With this information at hand, a search for the corresponding transporter could be undertaken.

3.2 Import of monocarboxylates

3.2.1 MctC is a pyruvate importer

In screening experiments performed by S. Ballan (2007), a mutant with a severe growth phenotype on pyruvate was identified. It was shown that the *icg/0833* knock-out mutant was not able to grow on pyruvate and a severe reduction in pyruvate uptake was observed. Here, the function of Cgl0833 was analyzed in more detail.

To exclude any side effects on the pyruvate dehydrogenase (PDH) in the *icg/0833* mutant, which might as well lead to the observed growth phenotype, PDH activity was measured in the wild type, a $\Delta aceE$ strain lacking the decarboxylating E1 subunit of the PDH and the *icg/0833* strain. As expected, no PDH activity was observed in the $\Delta aceE$ strain, but PDH activity rates were comparable in the wild type and the *icg/0833* mutant (table 3.1). Thus, a metabolic deficiency of the *icg/0833* strain at least at the level of the PDH could be excluded, confirming the idea of pyruvate uptake deficiency of the *icg/0833* strain. Considering that the *icg/0833* strain showed indistinguishable growth on glucose or lactate from the wild type (Ballan, 2007), other modifications of the central metabolism due to the integration of the pDrive plasmid were not expected.

Table 3.1 PDH activity in *C. glutamicum* WT, the $\Delta aceE$ and *icgI0833* strain. Specific activity was determined in crude cell extract and based on total protein content in the sample. n=3.

	specific activity [U/mg]
wild type	0.32 ± 0.04
$\Delta aceE$	0.00 ± 0.01
<i>icgI0833</i>	0.40 ± 0.02

In the next step, pyruvate import was characterized in the wild type and the *icgI0833* strain. Pyruvate is a carboxylic acid with a pKa of 2.39. At pH 7.0, 0.002% of pyruvate present in the medium is protonated and thus potentially diffusible. In order to determine the contribution of diffusion to pyruvate influx, uptake measurements with ^{14}C -labelled pyruvate were performed at the lowest possible physiological pH of 6.0 in order to detect both active and passive influx of pyruvate.

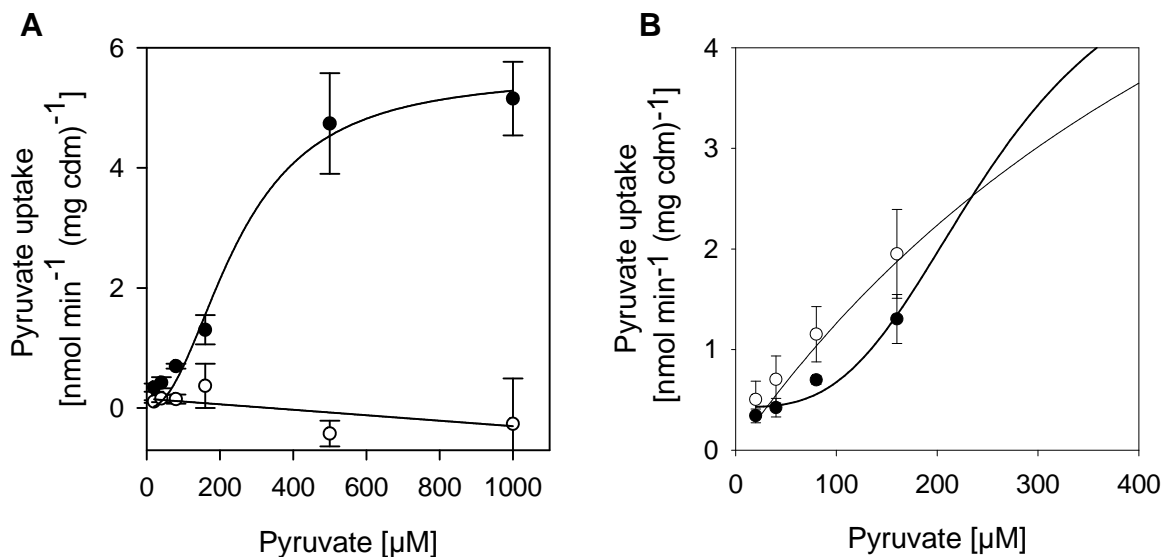


Fig. 3.2 A) Pyruvate uptake by *C. glutamicum* WT (closed symbols), and the *icgI0833* strain at pH 6.0 (open symbols). B) A close-up on pyruvate uptake in the presence of low pyruvate concentrations by *C. glutamicum* WT at pH 8.0 (open symbols) (Ballan, 2007) and at pH 6.0 (closed symbols). n=3.

As shown in Fig. 3.2A, the uptake of ^{14}C -labelled pyruvate was saturable at high substrate concentrations, reaching a V_{max} of $5.6 \pm 0.6 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$. The kinetics clearly indicated carrier mediated transport and could be best fitted according to the Hill equation whereby an apparent $K_{0.5}$ of $249 \pm 55 \text{ } \mu\text{M}$ was derived. The Hill coefficient of 2.1 ± 0.6 indicates cooperative substrate binding and this effect was more pronounced at pH 6.0 than at pH 8.0 (Fig. 3.2B).

No pyruvate uptake could be observed in the strain lacking Cgl0833 and even at high substrate concentration no pyruvate influx was detectable (Fig. 3.2A). This result supports the idea that the membrane protein Cgl0833 is absolutely indispensable for pyruvate import.

Passive influx of pyruvate was not observed, indicating that the plasma membrane of *C. glutamicum* is impermeable for this carboxylate.

To determine the optimal pH range of the carrier, pyruvate uptake measurements were performed at different physiological pH values. As depicted in Fig. 3.3, an optimum curve with a maximum at pH 7.0 was observed with decreasing uptake activities at more acidic and more alkaline pH. This demonstrates once more that diffusion does not play a role in pyruvate influx, since a decreasing pH would increase the share of the protonated and thus potentially membrane permeable acid in the medium and consequently the influx would increase at decreasing pH.

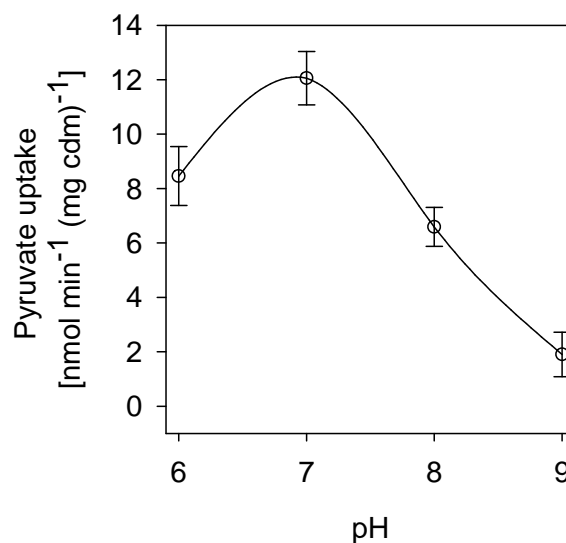


Fig 3.3 Pyruvate uptake by *C. glutamicum* WT at varying pH values in the presence of 1000 μ M pyruvate. n=3.

Summing up the results obtained so far, the normal activity of the PDH and the complete loss of pyruvate uptake ability in the *icgl0833* strain together with the fact that Cgl0833 is predicted to be a membrane protein with 13 transmembrane helices (TMHMM v.2.0) suggest that Cgl0833 is indeed a pyruvate transport system in *C. glutamicum*. Consequently, it will be further on designated MctC for **monocarboxylate transporter** of *C. glutamicum*. Moreover, since no pyruvate import was observed in the *mctC* knock-out strain, it seems to be the only pyruvate importer at the tested conditions.

3.2.2 Transport via MctC depends on the electrochemical proton potential

MctC is not predicted to have an ATP binding site, which suggests that it is a secondary active transporter. To prove this, pyruvate uptake was measured upon the addition of 20 μ M valinomycin and 5 μ M nigericin. Nigericin acts as an ionophore for H^+ and K^+ and valinomycin transports K^+ across the membrane. The combination of these compounds dissipates the membrane potential $\Delta\Psi$ and ΔpH and thus abolishes the driving force for secondary active transporters. The uptake rate in the uninhibited sample in the presence of

600 μM pyruvate was determined to be $1.96 \pm 0.16 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$ and only $0.17 \pm 0.18 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$ for cells with a collapsed membrane potential. This is a strong indication that MctC is indeed a secondary active transporter.

In order to determine the ion dependence of MctC, pyruvate uptake was measured in MES/Tris buffer with only residual amounts of Na^+ (10 μM) but no difference in uptake rates compared with pyruvate uptake in Na^+ containing buffer was observed (data not shown). To check the influence of H^+ ions on pyruvate transport, the membrane potential was abolished with 20 μM valinomycin in buffer containing 200 mM KCl. The transport of K^+ diminishes $\Delta\Psi$ and the residual ΔpH is the sole driving force for secondary active transport under these conditions. Internal pH values are kept constant at ca. 7.5 at external pH values of 6-9 (Follmann, 2008). Thus, a ΔpH could be set by variation of the external pH.

As depicted in Fig. 3.4, the cells were still able to import pyruvate after the collapse of the membrane potential but when a pH gradient was still present. On the other hand, the import ceased completely, when neither $\Delta\Psi$ nor ΔpH was present.

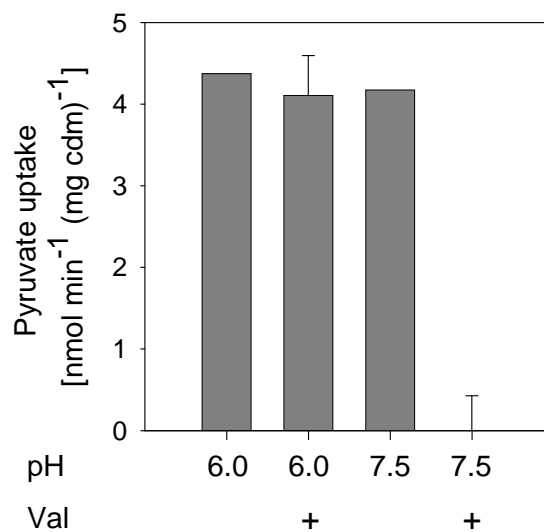


Fig 3.4 Pyruvate import by *C. glutamicum* at pH 6.0 and 7.5 upon the dissipation of the membrane potential by valinomycin. The measurements were performed in the presence of 600 μM pyruvate and 200 mM KCl. 20 μM valinomycin (Val) was added in the samples indicated by +. $n=3$ for valinomycin treated samples and $n=1$ for the controls.

As has already been indicated by the varying Hill coefficient in the dependence on external pH (Fig. 3.2B), this result demonstrates that MctC is a secondary active pyruvate importer depending on the electrochemical proton potential.

3.2.3 MctC imports the monocarboxylates acetate and propionate

To analyze the substrate spectrum of MctC, pyruvate uptake was measured in the presence of putative competitors in a 10-fold excess. Therefore, other monocarboxylates as lactate,

acetate and propionate as well as the dicarboxylate succinate and the amino acid alanine, which is structurally related to pyruvate, were chosen. The data is presented in table 3.2.

Table 3.2 Inhibition of pyruvate uptake by other carboxylates. 600 μM pyruvate was added along with the indicated substrates in 10-fold excess. Absolute pyruvate uptake in the control sample was $1.96 \pm 0.16 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$. $n=3$.

Substrate added	Pyruvate uptake rate [%]
Control	100 \pm 8
Lactate	86 \pm 18
Acetate	50 \pm 18
Propionate	38 \pm 30
Alanine	125 \pm 16
Succinate	104 \pm 3

This competition assay indicated that pyruvate uptake via MctC is not influenced by the presence of the structurally related monocarboxylate lactate. Also the presence of the dicarboxylate succinate or the amino acid alanine with a C_3 -backbone as pyruvate had no effect on pyruvate import. Of the tested substrates, only acetate and propionate inhibited pyruvate uptake severely, indicating possible binding to and translocation by MctC.

Although the observed inhibition of solute uptake can indicate which solutes may be bound by a carrier, not all competing substrates are transported. To prove that MctC indeed transports acetate and propionate, their uptake was monitored in the *C. glutamicum* ATCC 13032 and *imctC* strain.

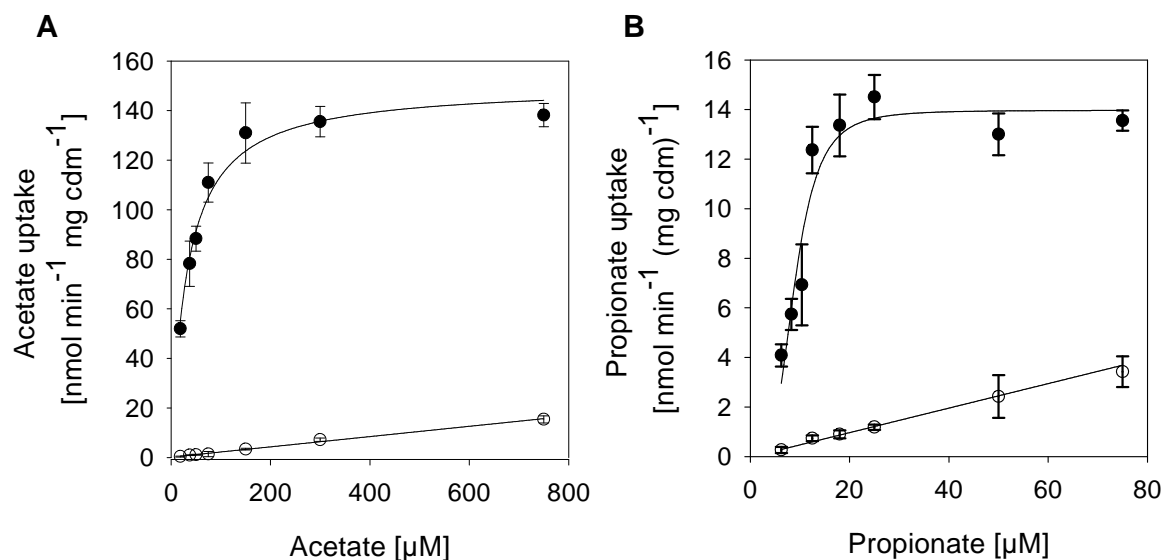


Fig. 3.5 A) Acetate and B) propionate uptake of *C. glutamicum* WT (closed symbols) and *imctC* strain (open symbols) at pH 8.0. $n=3$.

As can be seen from Fig. 3.5, acetate and propionate are imported in the *C. glutamicum* ATCC 13032 strain. The transport activity is saturable with increasing substrate concentrations, arguing for protein mediated transport. Plotting acetate uptake rates against substrate concentration showed that the data was best fitted by the Michaelis-Menten equation. A K_m of $32 \pm 4 \mu\text{M}$ and a V_{\max} of $150 \pm 5 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$ for acetate uptake can be calculated from this data. For propionate, the data obtained was best fitted according to the Hill equation rather than to Michaelis-Menten and a $K_{0.5}$ of $9 \pm 1 \mu\text{M}$ and a Hill coefficient of 3.65 ± 1.07 was calculated. A V_{\max} of $14 \pm 1 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$ was observed in this experiment.

Furthermore, acetate and propionate import ceased in the *imctC* strain. The remaining uptake activity increased linearly with increasing substrate concentrations. Acetate and propionate have a comparable pK_a of 4.75 and 4.87, respectively. Consequently, at pH 6.0, 5% of acetate and 7% of propionate are protonated and thus might diffuse passively through the cytoplasmic membrane. Raising the pH by one unit leads to dissociation of the acid and approximately 10 times less acid is present in its protonated form. The unsaturable uptake behavior indicates that the remaining uptake activity in the *imctC* strain was due to diffusion of the substrate into the cells. To determine the contribution of the active transporter and of diffusion to overall acetate and propionate uptake, uptake of these substrates was monitored in the physiological pH range of 6.0-9.0. Uptake rates were determined in the presence of $300 \mu\text{M}$ acetate or $25 \mu\text{M}$ propionate at various pH. The result is depicted in Fig. 3.6.

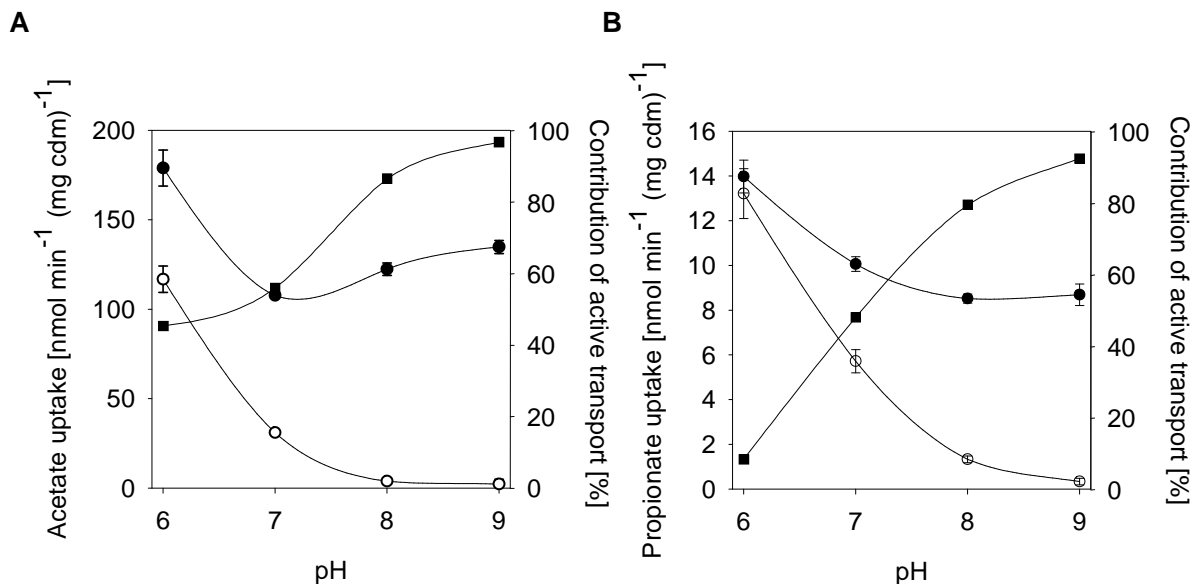


Fig. 3.6. A) Acetate and B) propionate uptake in *C. glutamicum* WT (closed circles) and the *imctC* strain (open circles) as measured at different pH values. Measurements were performed in the presence of $300 \mu\text{M}$ acetate or $25 \mu\text{M}$ propionate. The share of active transport to overall influx was calculated from these data (squares). $n=3$.

For both substrates, a decrease of uptake rates with increasing pH values was observed in the wild type and the *imctC* strain. Interestingly, the uptake rate of acetate did not decrease gradually in the wild type, but showed a minimum at pH 7.0 and slowly increased at higher pH, indicating a pH optimum at $\text{pH} \geq 9.0$, as has been previously observed by Ebbighausen *et al.* (1991a). At lower pH values uptake of both substrates was observed in the *imctC* strain; for propionate almost the same uptake rates were determined as for the wild type. On the other hand, increasing the pH led to an almost complete cessation of acetate or propionate uptake in the *imctC* strain, indicating that MctC is indeed required for acetate and propionate uptake at higher pH values. This supports the idea that MctC is the only acetate and propionate importer in *C. glutamicum* under these experimental conditions.

From the presented data, the contribution of the active transporter MctC to the overall influx of the diffusible substrates acetate and propionate can be calculated. As indicated in Fig. 3.6 at pH 9.0 and at the particular substrate concentrations, almost 100% of substrate uptake is due to carrier mediated transport, whereas this share decreases at lower pH values and becomes even neglectable for propionate uptake at pH 6.0. This demonstrates that acetate and propionate diffuse into the cell even without the presence of an active uptake system and that the influx of acetate and propionate cannot be avoided at lower pH.

Under natural conditions, only μM amounts of acetate or propionate are found in soil (Rothfuss and Conrad, 1993) and although the pH in soil is rather acidic, the limited amount of the carboxylates represents a selective pressure on a bacterial population. Therefore species with an uptake system should have a growth advantage. To test this idea, *C. glutamicum* WT and the ΔmctC strain were grown in microtiter plates at low acetate concentrations. In order to demonstrate the role of MctC, uptake via diffusion was limited in the experiment by setting the pH to 8.5.

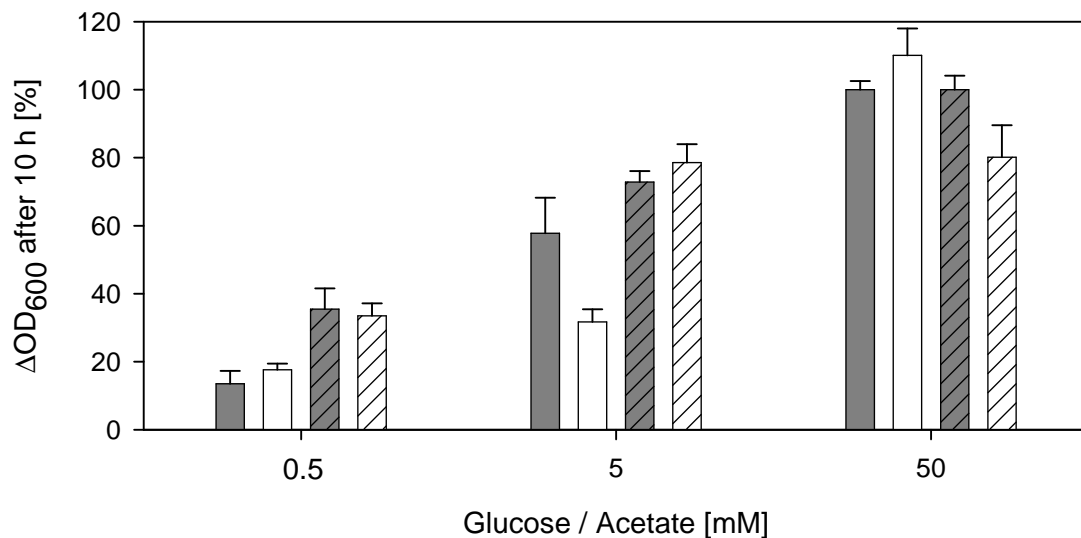


Fig. 3.7 Growth of *C. glutamicum* WT (filled bars) and $\Delta mctC$ (open bars) on different amounts of acetate (empty bars) or glucose (striped bars) at pH 8.5. Growth was monitored in microtiter plates and the increase in optical density (OD_{600}) after 10 h monitoring time is given. The value for wild type growth on 50 mM acetate ($\Delta OD_{600} = 0.3$) or glucose ($\Delta OD_{600} = 0.8$) was set to 100%. $n=4$.

As shown in Fig. 3.7, in the presence of 0.5 mM substrate, only minute growth of both strains was observed on acetate and slightly better one on glucose. In the presence of 50 mM substrate, both strains showed substantial and comparable growth on both substrates. In the presence of 5 mM glucose, both strains grew equally well. However, in the presence of 5 mM acetate, only the wild type strain showed substantial increase in biomass as compared to the value on 0.5 mM acetate. Growth of the $\Delta mctC$ strain was severely restricted at these low acetate concentrations.

Thus, the biological relevance of MctC is pyruvate import as well as substantial contribution to acetate and propionate uptake especially at higher pH values and low substrate concentrations, as under natural conditions. The increased velocity of substrate uptake due to the presence of an active transporter is a beneficial trait for the cell in nutrient competition with other organisms. On the other hand, the passive influx of acetate causing acidic stress has to be considered in biotechnological applications.

3.2.4 *mctC* is regulated by the presence of monocarboxylates

As outlined above, the presence of MctC is of vital importance for growth ability on pyruvate and beneficial for growth on the membrane permeable substrates acetate and propionate at higher pH. It was therefore of peculiar interest, whether MctC activity or transcription of the corresponding gene is dependent on the presence of carboxylates. In the first place the activity of the transporter was assayed after pre-cultivation of *C. glutamicum* on glucose, lactate, acetate, or pyruvate and acetate uptake measurements were performed in the presence of 300 μ M acetate.

As summarized in table 3.3, acetate uptake activity was highest after pre-cultivation on acetate, but also significantly higher after pre-cultivation on the other carboxylates than after cultivation on glucose. This result confirmed the assumption that MctC activity is modulated by the presence of its substrates and surprisingly also by the presence of the monocarboxylate lactate, which is not transported by MctC. This suggested regulation on transcriptional level.

Table 3.3 Acetate uptake rate upon the pre-cultivation of *C. glutamicum* on different carbon sources. The acetate uptake rate after growth on glucose was $85 \pm 18 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$ and was set to 100%. Measurements were performed in the presence of 300 μM acetate at pH 8.0. n=3.

	Acetate uptake rate [%]		
Glucose	100	\pm	22
Lactate	139	\pm	6
Acetate	164	\pm	16
Pyruvate	131	\pm	6

3.2.5 *mctC* and *cgI0832* form a transcriptional unit and are regulated by RamA and RamB

In order to identify potential regulators of *mctC* transcription, the genetic localization of *mctC* was analyzed first. Only 6 bp upstream of *mctC* is located *cgI0832*, which is predicted to encode an 113 amino acids small protein with two transmembrane helices (TMHMM v.2.0 predictor (Krogh *et al.*, 2001)). In order to test, whether these two might form a functional unit, their co-transcription in an operon was analyzed. Therefore, total RNA from *C. glutamicum* was isolated and reverse transcribed into cDNA after preceding DNA digestion. This cDNA was used as template in subsequent PCR reaction combining primers that would amplify the *cgI0832-mctC* operon or only the *mctC* cDNA. As a control, primers amplifying a fragment in *mctC* were applied. The result is depicted in Fig. 3.8.

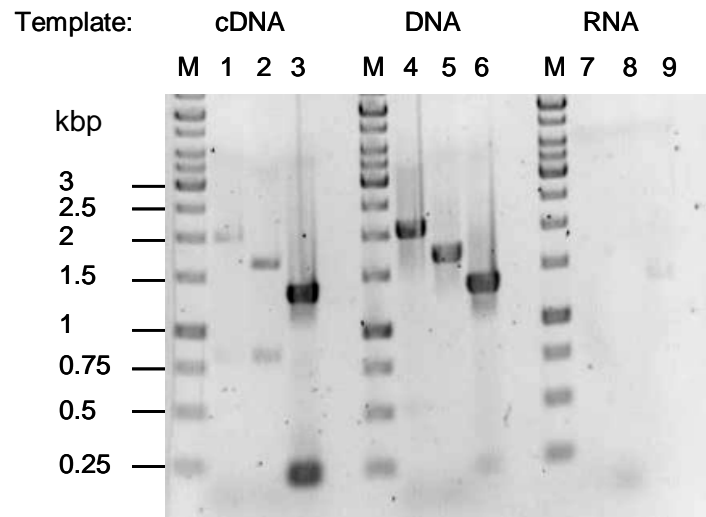


Fig. 3.8 Operon structure of *cgI0832* and *mctC*. The RT-PCR was performed with RNA, DNA and cDNA synthesized with random hexamers. Detection of fragments was performed by PCR with primers amplifying *cgI0832-mctC* (lane 1, 4, 7), *mctC* only (lane 2, 5, 8) and an internal fragment of *mctC* as control (lane 3, 6, 9).

As can be seen from Fig. 3.8, all three primer combinations led to an amplicon when cDNA but not RNA was used as template (DNA was used as control). The product obtained using primers for *cgI0832-mctC* amplification (lane 1 in Fig. 3.8) points out that these two genes are indeed transcribed in an operon.

Regarding the promoter region of *cgI0832-mctC*, binding sites of several transcriptional regulators were identified (Fig. 4.2). Considering the fact that MctC plays an important role in acetate metabolism, its regulation by the major regulators of acetate metabolism RamA and RamB was of special interest. RamA is the major activator of genes encoding enzymes required for acetate utilization, whereas RamB is their repressor. Indeed, regarding the expression of *cgI0832-mctC* in a $\Delta ramA$ strain, both were found to be induced (Cramer, unpublished). Furthermore, promoter test studies performed by D. Emer in *C. glutamicum* WT and the $\Delta ramA$ or $\Delta ramB$ strains carrying the promoter test vector pET2 with the promoter region of *cgI0832* fused to the promoterless CAT gene showed also transcription dependence of *cgI0832* on the carbon source and on the presence of RamA and RamB. The results are summarized in table 3.4.

Table 3.4 Specific CAT activities of *C. glutamicum* strains carrying the 477 bp *cgI0832-mctC* promoter fragment in pET2. The cells were grown in minimal medium (MM) containing different carbon sources. n. g. = no growth. Data were obtained and courteously provided by D. Emer.

<i>C. glutamicum</i> strain	specific CAT activity [U/mg protein] in MM with			
	glucose	acetate	pyruvate	acetate + propionate
WT (pET2- <i>mctC_P</i>)	0.56 ± 0.05	0.62 ± 0.10	1.13 ± 0.07	1.33 ± 0.23
$\Delta ramA$ (pET2- <i>mctC_P</i>)	0.04 ± 0.01	n.g.	n.g.	n.g.
$\Delta ramB$ (pET2- <i>mctC_P</i>)	0.95 ± 0.07	0.85 ± 0.22	1.45 ± 0.03	0.67 ± 0.17

As summarized in table 3.4, the promoter activity of the wild type was higher upon cultivation on pyruvate and on the mixture of acetate and propionate. The absence of RamA in the $\Delta ramA$ strain caused a complete loss of promoter activity and the lack of MctC disabled growth on pyruvate. Growth inability on acetate or acetate + propionate was most likely caused by the absence of acetate utilizing enzymes, but was not tested here. The absence of the repressor RamB was expected to cause a de-repression of *mctC* transcription. However, though the promoter activity was higher in the $\Delta ramB$ strain than in the wild type, a pyruvate dependent induction was still observed, indicating the presence of another repressor whose action is modulated by the presence of pyruvate.

All in all, both regulators obviously mediate the regulation of *cgI0832-mctC* transcription in dependence on the applied carbon source. However, the discrepancy between the same expression level of *cgI0832* upon cultivation on glucose and acetate and increased MctC activity upon pre-cultivation on acetate as well as the incomplete de-regulation in $\Delta ramB$ cells require further investigations considering additional regulatory mechanisms involved in carboxylate metabolism.

3.3 Import of lactate

3.3.1 Lactate import is an active process

Previously described experiments showed that the transport of the monocarboxylates pyruvate, acetate, and propionate is mediated by MctC. As shown in 3.1, the laboratory strain *C. glutamicum* ATCC 13032 is also able to utilize L-lactate as further monocarboxylate for growth. However, no indication for L-lactate transport by MctC was given, suggesting a different transport mechanism, which was elucidated in the following experiments. In order to determine, whether lactate influx requires an active import system and to characterize its features, uptake measurements with ^{14}C -labelled lactate were performed. Moreover,

transcriptome data suggested Cgl2917 as a putative lactate permease (Stansen *et al.*, 2005). Therefore, the *icgl2917* knock-out mutant was also tested for its lactate import ability.

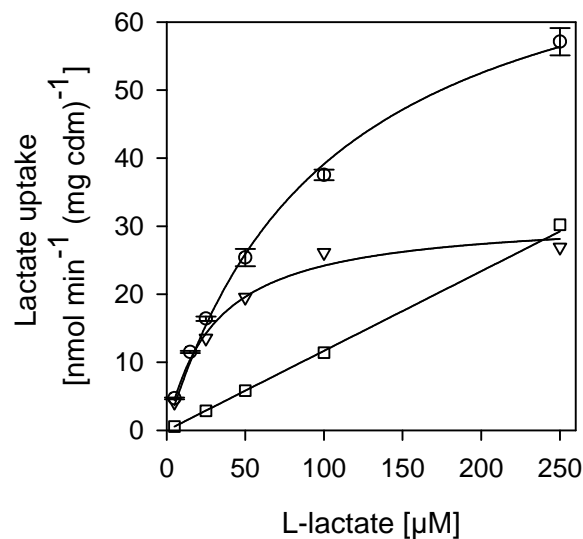


Fig. 3.9 L-lactate uptake of *C. glutamicum* (circles) and the *icgl2917* strain (squares) in the dependence on L-lactate concentration. Additionally, the *icgl2917* values were subtracted from wild type values and the resulting values are plotted (triangles). $n=3$ for wild type and $n=1$ for *icgl2917*.

As depicted in Fig. 3.9, L-lactate uptake by *C. glutamicum* wild type was saturable with increasing substrate concentrations and could be best fitted according to Michaelis-Menten equation, indicating the presence of a transport system. L-lactate import rates increased linearly with increasing substrate concentration in the *icgl2917* strain. Although at first glance, the transport kinetics of *icgl2917* argue for lactate diffusion, this idea was challenged by the fact that the accumulation of radiolabelled substrate was independent of the specific activity of L-lactate in the assay for the *icgl2917* strain, resulting in a linear increase of the uptake rate (Fig. 3.9). It can therefore be assumed that this increase was due to the influx of a substrate which was not L-lactate. Since this holds true also for wild type cells, the kinetics for wild type were corrected by subtraction of the *icgl2917* import rate values in order to determine kinetic parameters for L-lactate uptake. Hereupon, an apparent K_m value of 30 ± 6 μM was derived and a V_{\max} of 32 ± 2 $\text{nmol min}^{-1} (\text{mg cdm})^{-1}$ was observed in this experiment.

The severe reduction of L-lactate uptake in the *icgl2917* strain supports the idea that Cgl2917 is a lactate import system. However, it has to be considered, that the insertion in *cgl2917* caused polar effects on the adjacently encoded lactate dehydrogenase LldD (Stansen *et al.*, 2005). Thus, the impaired uptake activity of *icgl2917* might have also resulted by the absence of the L-lactate utilizing enzyme. To test this hypothesis, the knock-out of *lldD* needs to be complemented in the *icgl2917* strain and the resulting strain needs to be tested for L-lactate uptake activity. Although biochemical data on this strain are missing,

it regained growth ability on L-lactate as sole carbon source (Stansen *et al.*, 2005), putting the idea of Cgl2917 being a lactate importer into question.

To further characterize lactate import, the mechanism of lactate uptake was assessed. The dependence on ATP was addressed by the application of vanadate, that on membrane potential by CCCP or valinomycin/nigericin and that on Na⁺ ions by Na⁺ depletion of the buffer. As summarized in table 3.5, the application of vanadate caused only minor inhibition, but lactate import ceased completely when the membrane potential was collapsed. This suggests that lactate import occurs via a secondary active transporter. However, a clear statement on its ion dependence cannot be drawn from this experiment, since trace amounts of Na⁺ (10 µM) were still present in the Na⁺-depleted sample and thus would be sufficient to drive transport provided that the carrier has a high affinity for Na⁺. Thus, further experiments concerning ion dependence of L-lactate import are required.

Table 3.5 Energetization of L-lactate uptake by *C. glutamicum*. Uptake of L-lactate was measured in the presence of 250 µM L-lactate and different inhibitors. Uptake rates of 54 nmol min⁻¹ (mg cdm)⁻¹ were determined in the control sample and set as 100%. w/o = without. n=1.

	Uptake rate [%]
Control	100
w/o Na ⁺	105
CCCP	22
Valinomycin+Nigericin	2
Vanadate	64

In summary, the data shown here clearly indicate that lactate import requires the presence of a secondary active transport system. However, a clear statement on Cgl2917 involvement in L-lactate import could not be drawn from the presented experiments. The regained growth ability of the *icgl2917* strain upon the introduction of a constitutively expressed LldD indicated the presence of a further lactate importer besides Cgl2917.

3.3.2 DctA is a lactate importer candidate

As described in the preceding chapter, the import of L-lactate is mediated by a secondary active transporter. In order to identify the uptake system, a screening assay of the site-directed insertion mutant collection that was present at that time was carried out on minimal medium agar plates containing L-lactate as sole carbon source. A mutant being defective in the lactate import system was expected to show reduced growth. Of the 88 tested strains, only *icgl2595* and *icgl2917* showed a particular phenotype (Fig. 3.10). A subsequent test in liquid media confirmed this result for the *icgl2595* strain (Fig. 3.11A). *icgl2917* was not further assessed due to polar effects on the lactate converting LldD upon the disruption of *cgl2917* (Stansen *et al.*, 2005).

Results

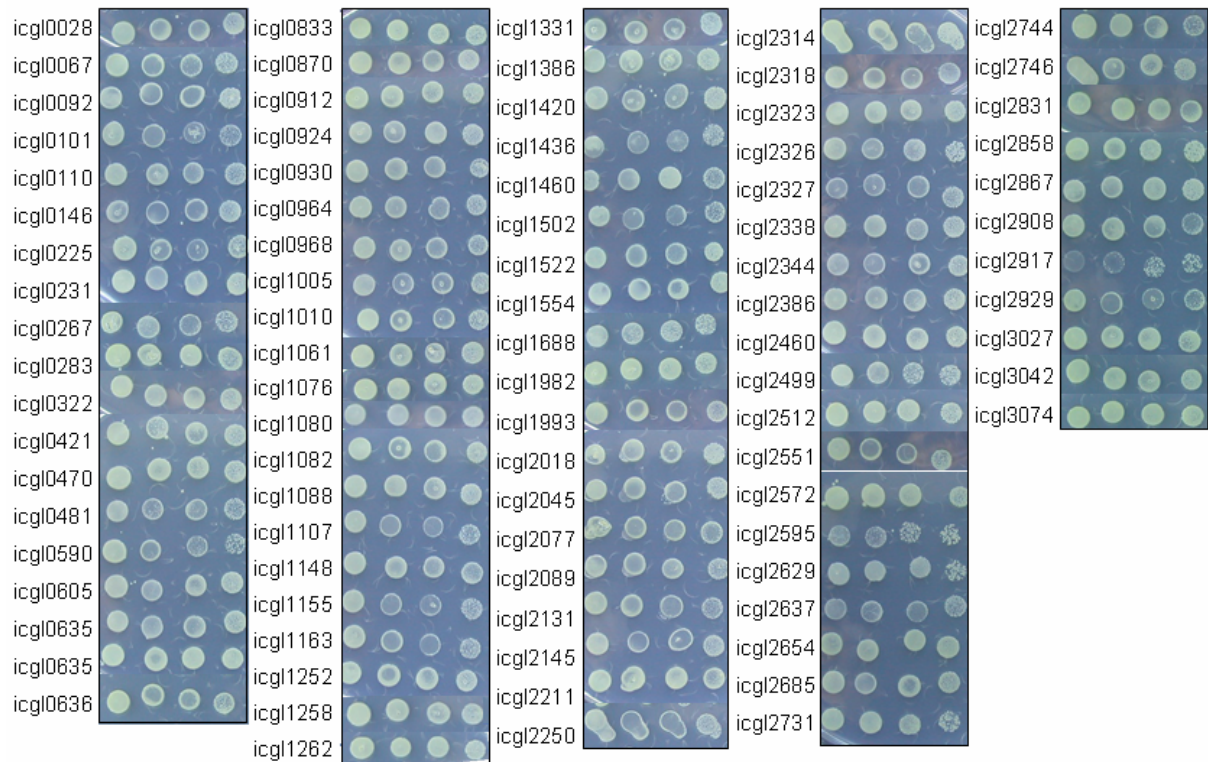


Fig. 3.10 Growth of *C. glutamicum* insertion mutant collection on L-lactate containing MM1-plates. 5 μ l of a cell culture of an OD_{600} of 1, 0.1, 0.01, and 0.001 were spotted on agar plates and growth was evaluated after 48 h at 30°C.

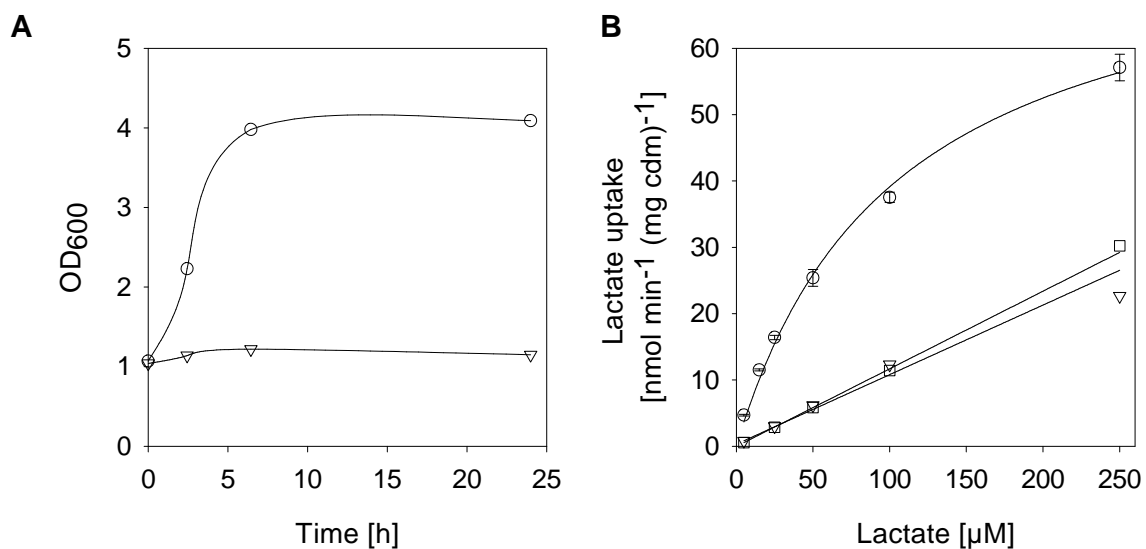


Fig. 3.11 A) Growth of *C. glutamicum* wild type (circles) and *icgl/2595* (triangles) on L-lactate as sole source of carbon and energy. B) L-lactate uptake by *C. glutamicum* wild type (circles) and *icgl/2595* (triangles). L-lactate uptake of *icgl/2917* has already been presented in Fig. 3.9 and is shown for comparison (squares). $n=3$ for L-lactate uptake of the wild type and $n=1$ for other measurements.

The result for *icgl/2595* was furthermore validated by lactate uptake measurements, where comparable uptake rates to the *icgl/2917* strain were observed (Fig. 3.11B). As for *icgl/2917*, the accumulation of radioactive substrate was independent of the L-lactate concentration. This was taken as an indication for a contamination of the radioactive probe with an

unknown substrate. Besides, even if L-lactate diffused into the cells, it was not sufficient for biomass production, as indicated by the growth phenotype of the *icgl2595* strain (Fig. 3.11A).

Cgl2595 is annotated as the aerobic dicarboxylate transporter DctA. Regarding the genetic localization of the corresponding gene, an operon structure with a deacetylase can be assumed. However, no genes coding for lactate utilizing enzymes are located in the vicinity of *dctA*, in contrast to *cgl2917*, which is adjacent to *lldD*, encoding the L-lactate dehydrogenase (Fig. 3.12). Polar effects of an insertion in *dctA* on lactate utilizing enzymes are therefore not likely.

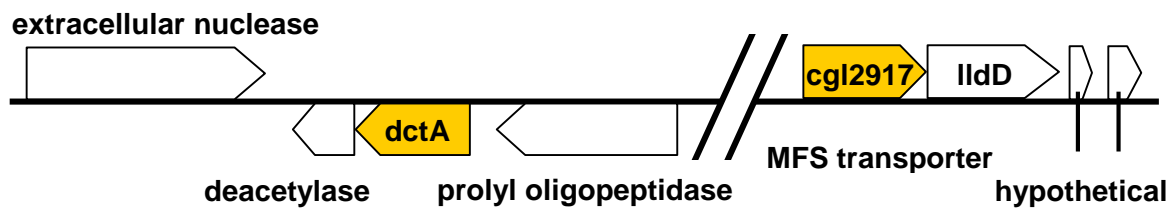


Fig. 3.12 Genetic environment of *dctA* and *cgl2917*. Genes encoding proteins with transmembrane domains are indicated by filled arrows. 360 kb in between were skipped, as indicated by vertical lines.

The data collected so far on DctA in *C. glutamicum* implicated its involvement in L-lactate uptake. Its knock-out had deleterious effects on lactate uptake ability of *C. glutamicum* and consequently on growth on this carboxylate. However, whether DctA indeed imports L-lactate remains to be tested in further experiments, considering complementation studies in the first place. For homologues of DctA from other organisms as e.g. *E. coli*, its involvement in aerobic dicarboxylate uptake was shown (Kay and Kornberg, 1971). Whether this holds true for DctA from *C. glutamicum* was analyzed and is described in the following chapter.

3.4 Import of dicarboxylates

As observed previously, the laboratory *C. glutamicum* strain does not grow on dicarboxylates such as succinate, fumarate or malate (conf. 3.1). However, prolonged incubation of cells on succinate, fumarate or malate led to isolation of spontaneous mutants which utilized these substrates for growth (Youn *et al.*, 2008). The mutant isolated on succinate was further on designated SSM (succinate spontaneous mutant), the one on fumarate FSM (fumarate spontaneous mutant) and the one on malate MSM (malate spontaneous mutant). Transcriptomic analyses revealed altered transcription rates of several genes in the SSM and FSM strain. Among those, *cgl0225* is annotated as a DASS (divalent anion:sodium symporter) type transporter encoding gene. Unlike SSM and FSM, the MSM strain showed *inter alia* increased transcription rates of *cgl2595*, which is annotated as a Na^+/H^+ dicarboxylate symporter encoding gene (*dctA*). Both putative transporters were thereupon analyzed regarding their function and biochemical properties.

3.4.1 DctA is a dicarboxylate importer of *C. glutamicum*

To prove, whether DctA is indeed a dicarboxylate importer in *C. glutamicum*, the corresponding gene was expressed in *C. glutamicum* ATCC 13032 and uptake measurements were performed with ^{14}C -labelled succinate, fumarate, and malate. The results are depicted in Fig. 3.13.

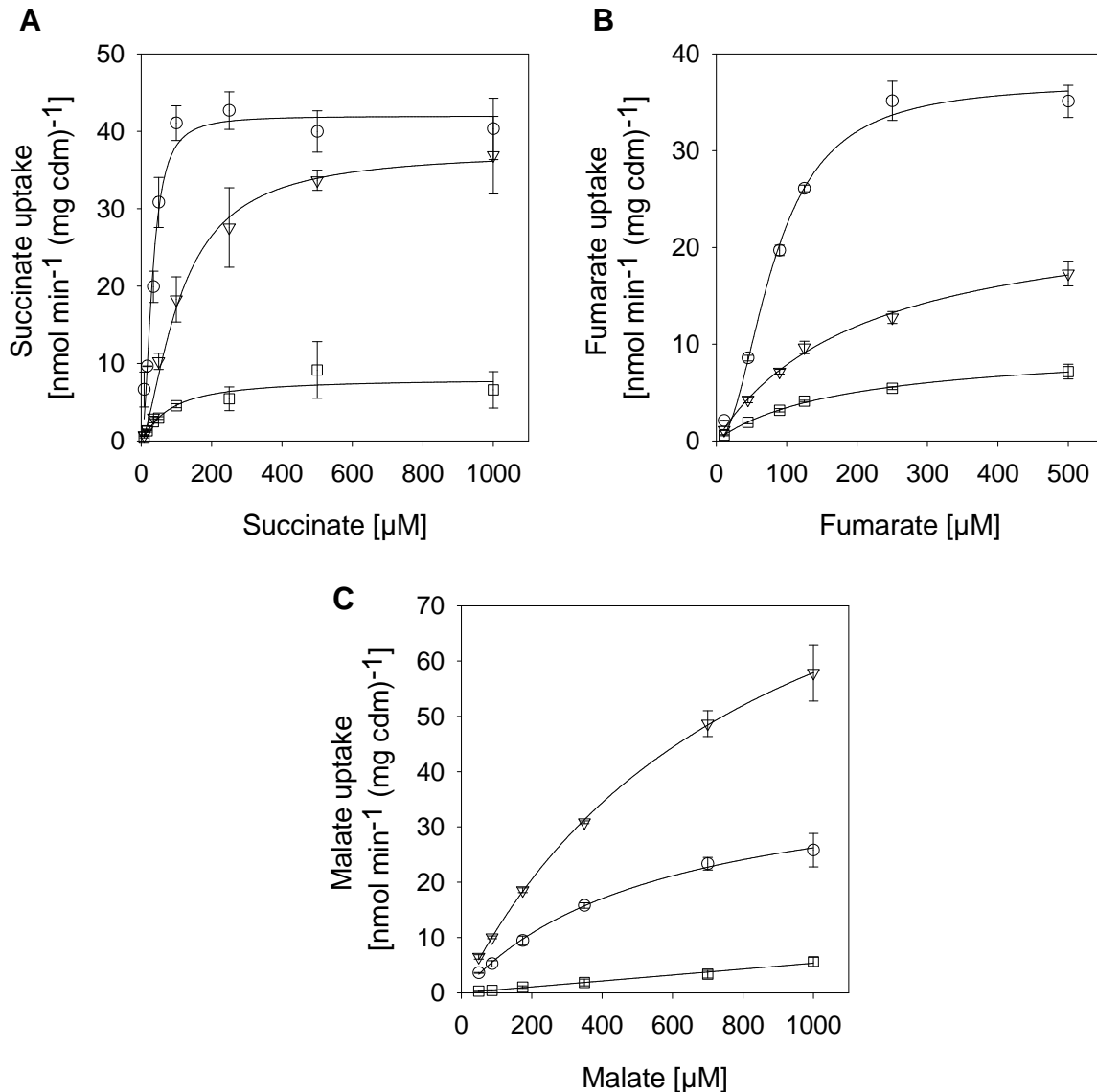


Fig. 3.13 Uptake of A) succinate, B) fumarate, C) L-malate by *C. glutamicum* ATCC 13032 (squares), *C. glutamicum* (pVWEx1-dctA) (triangles), SSM (circles in A), FSM (circles in B), and MSM (circles in C) as measured with ^{14}C -labelled substrates. $n=3$.

Dicarboxylate uptake was accelerated in the spontaneous mutants as compared to the wild type. The introduction of *dctA* also caused enhanced dicarboxylate uptake activity. This result strongly suggests that DctA is a dicarboxylate importer of *C. glutamicum*. To determine kinetic parameters for DctA transport, the uptake rates determined in the wild type were subtracted from the measured rates in the pVWEx1-dctA strain, since the presence of another dicarboxylate transporter could not be excluded. The resulting values could be best

fitted according to Michaelis-Menten equation and kinetic data were derived. Thus, an apparent K_m of $119 \pm 25 \mu\text{M}$ for succinate, $226 \pm 89 \mu\text{M}$ for fumarate, and $731 \pm 153 \mu\text{M}$ for L-malate were calculated and maximal uptake rates of $29 \pm 2 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$ for succinate, $15 \pm 3 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$ for fumarate and $91 \pm 9 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$ for L-malate were observed under the tested conditions.

However, large differences between the K_m values for DctA and the values determined in the spontaneous mutants were observed. The uptake kinetics of the SSM and FSM strain were best fitted according to the Hill equation, resulting in $K_{0.5}$ of $30 \pm 4 \mu\text{M}$ and $79 \pm 7 \mu\text{M}$, respectively. Uptake kinetics of the MSM strain were fitted according to the Michaelis-Menten equation and resulted in an apparent K_m of $305 \pm 90 \mu\text{M}$.

Thus, the activity of DctA could not be confirmed for any of the tested spontaneous mutants, although the transcriptional analysis indicated the presence of DctA in the MSM strain. Whether increased *dctA* expression can be correlated to increased DctA presence in the MSM strain should be analyzed in further studies regarding the proteome of the MSM strain. However, since the expression of *dctA* in the wild type caused a clear import of dicarboxylates, the function of DctA as a succinate, fumarate, and L-malate importer seems to be proven.

3.4.2 Transport via DctA depends on the electrochemical proton potential

In order to determine the driving force for DctA mediated dicarboxylate transport, L-malate uptake measurements were performed in buffer with only residual amounts of Na^+ ($10 \mu\text{M}$) but no difference in uptake rates compared with L-malate uptake in Na^+ containing buffer was observed (data not shown).

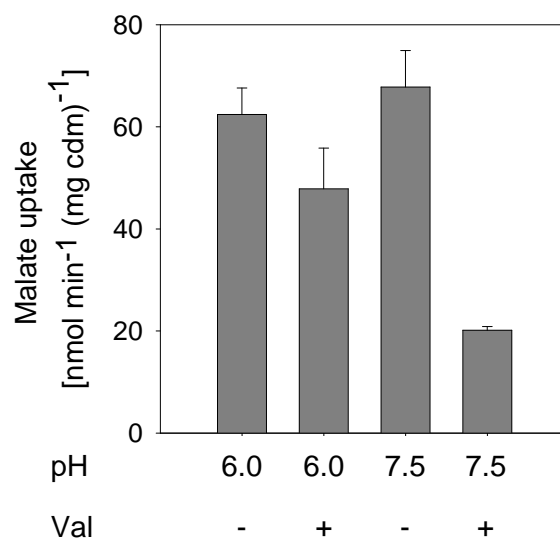


Fig 3.14 L-malate import by *C. glutamicum* (pVWEx1-*dctA*) at pH 6.0 and 7.5 upon the dissipation of the membrane potential by valinomycin. The measurements were performed in the presence of $700 \mu\text{M}$ L-malate and 200 mM KCl. $20 \mu\text{M}$ valinomycin (Val) was added in the samples indicated by +. $n=3$.

To test, whether L-malate uptake depends on the proton potential, L-malate uptake was measured upon the dissipation of $\Delta\Psi$ and the concomitant dissipation of $\Delta\Psi$ and ΔpH , assuming an internal pH of 7.5. As shown in Fig. 3.14, the cells were still able to import L-malate after the collapse of the membrane potential but in the presence of a pH gradient. On the other hand, the import was severely reduced, when neither $\Delta\Psi$ nor ΔpH was present. This result supports the idea, that DctA is a secondary active dicarboxylate importer depending on the proton potential.

3.4.3 DctA has a broad substrate spectrum

To determine whether DctA accepts further substrates besides succinate, fumarate and L-malate, L-malate uptake was measured in *C. glutamicum* pVWEx1-*dctA* in the presence of putative competitors in 100-fold excess. As potential substrates were regarded the intermediates of the TCA, L-glutamate and L-aspartate as further dicarboxylates and glyoxylate, L-lactate, and pyruvate as monocarboxylates. As summarized in table 3.6, many structurally different substrates inhibited L-malate uptake by DctA. Those were the tested intermediates of the TCA, glyoxylate, L-lactate, and L-aspartate, although the inhibition caused by the latter as well as those caused by citrate was not that pronounced as the complete inhibition caused by C₄-dicarboxylates, 2-oxoglutarate, and glyoxylate. Moreover, the addition of L-lactate also caused slight inhibition of L-malate uptake, supporting the previously discussed idea of DctA being the L-lactate importer in *C. glutamicum* (conf. 3.3.2).

Table 3.6 L-malate uptake by *C. glutamicum* pVWEx1-*dctA* strain upon the addition of several organic acids. Competing substrates were added in 100 fold excess in the presence of 700 μM L-malate, with the exception of fumarate, which was added in 10 fold excess due to solubility reasons. Uptake rates are given as percentage of the control rate of $62 \pm 2 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$. n=3.

Substrate added	L-Malate uptake [%]
Control	100 \pm 3
Citrate	49 \pm 7
Isocitrate	76 \pm 1
2-Oxoglutarate	0 \pm 1
Succinate	5 \pm 2
Fumarate	4 \pm 2
Oxaloacetate	1 \pm 1
Glyoxylate	4 \pm 2
Pyruvate	77 \pm 2
L-Lactate	68 \pm 1
L-Glutamate	87 \pm 3
L-Aspartate	58 \pm 1

However, this inhibition study can be taken only as a first idea of the substrates DctA might accept. To prove the actual transport of these substrates by DctA, detailed uptake studies or growth experiments on the respective substrate are required.

3.4.4 DccT is another dicarboxylate importer of *C. glutamicum*

In contrast to the spontaneous mutant isolated upon growth on L-malate, which showed increased transcription of *dctA*, mutants isolated on succinate and fumarate showed increased transcription of *cgl0225*, which encodes a membrane protein. To prove the function of Cgl0225 as a dicarboxylate importer, the corresponding gene was expressed in *C. glutamicum* ATCC 13032 and uptake measurements were performed with ^{14}C -labelled succinate, fumarate, and malate.

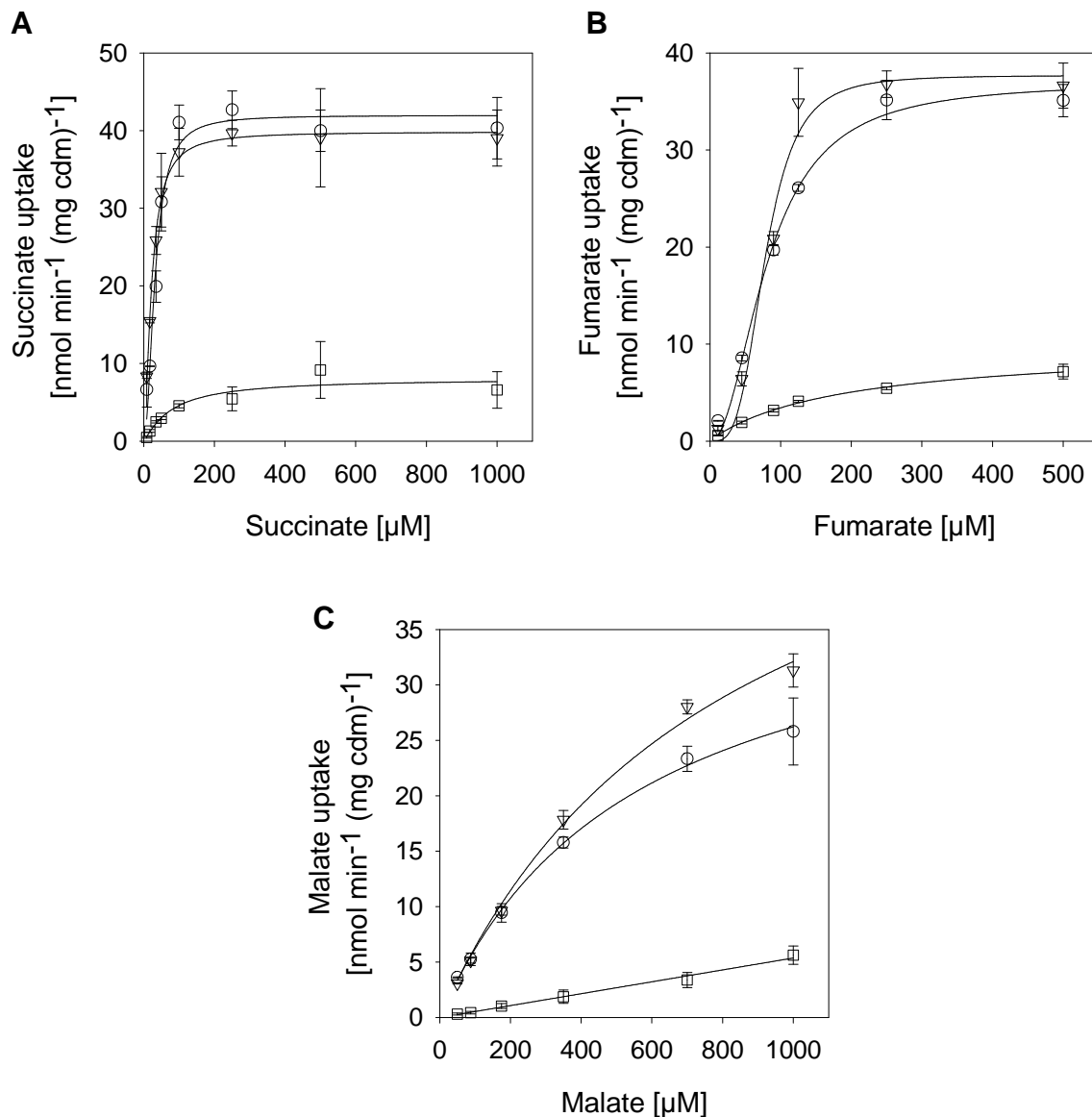


Fig. 3.15 Uptake of A) succinate, B) fumarate, C) L-malate by *C. glutamicum* ATCC 13032 (squares), *C. glutamicum* pVWEx1-*cgl0225* (triangles), SSM (circles in A), FSM (circles in B), and MSM (circles in C) as measured with ^{14}C -labelled substrates. $n=3$.

As shown in Fig. 3.15, both the spontaneous mutants and *C. glutamicum* pVWEx1-*cgl0225* showed substantial dicarboxylate uptake, which was saturable at higher substrate concentrations, indicating the presence of an active transport system. Therefore, the designation DccT for **d**icarboxylic acid **c**orynebacterial **t**ransporter will be followed from here on referring to the gene product of *cgl0225*. However, also the *C. glutamicum* ATCC 13032 strain showed some residual uptake activity for each of the given substrates. Since it could not be excluded, that another dicarboxylate importer was active in the wild type and whose activity was superimposed in the spontaneous mutants and the overexpression strain, the uptake rates of the wild type were subtracted from the data obtained for the mutants in order to determine the kinetic values for the particular transport system. The hereby resulting values for malate uptake could be best fitted according to the Michaelis-Menten equation, but the uptake rates determined for succinate and fumarate needed to be fitted by the Hill equation and Hill coefficients ≥ 2 indicated cooperative binding of the translocated substrates. Hereupon, the data obtained for malate uptake was also fitted according to Hill resulting in Hill coefficients of approx. 1. The kinetic data are summarized in table 3.7.

Table 3.7 Kinetic data on dicarboxylate uptake by *C. glutamicum* pVWEx1-*dccT* and the SSM, FSM, and MSM strains. Uptake rates observed in *C. glutamicum* ATCC 13032 were subtracted from the corresponding uptake rates in the mutants in order to calculate the $K_{0.5}$ and V_{\max} values. $K_{0.5}$ is given in μM , V_{\max} is given in $\text{nmol min}^{-1} (\text{mg cdm})^{-1}$.

	$K_{0.5}$	V_{\max}	Hill-coefficient
Succinate uptake			
SSM	30 ± 4	35 ± 2	2.2 ± 0.7
pVWEx1- <i>dccT</i>	19 ± 2	33 ± 1	1.8 ± 0.3
Fumarate uptake			
FSM	79 ± 7	30 ± 2	2.4 ± 0.5
pVWEx1- <i>dccT</i>	80 ± 7	31 ± 2	4.6 ± 1.7
Malate uptake			
MSM	305 ± 90	26 ± 4	1.2 ± 0.2
pVWEx1- <i>dccT</i>	361 ± 87	33 ± 4	1.3 ± 0.2

Comparing the overexpression strains and the spontaneous mutant strains, it is noticeable that the uptake behavior of *C. glutamicum* pVWEx1-*dccT* corresponds to the uptake behavior of the SSM strain considering succinate uptake, FSM considering fumarate uptake and MSM considering malate uptake. This suggested the idea that the increased transcript level and thus increased number of DccT molecules promoted uptake and thus growth ability on succinate, fumarate, and malate. Regarding its affinities towards the different substrates, DccT can be characterized as a succinate-fumarate-malate importer. A striking

difference is the different Hill coefficient for each of these substrates. However, the accurate determination of the number of co-translocated molecules requires further experiments with different concentrations of the co-substrate. Therefore, the co-substrate has to be identified first.

3.4.5 DccT requires Na^+ for transport

DccT is annotated as a DASS-type (divalent anion:sodium symporter) transporter. In order to test Na^+ dependence of DccT, uptake measurements were carried out with the SSM strain in the presence of 800 μM succinate as the preferred substrate and with varying Na^+ concentrations. Hereupon, a strong dependence on the presence of Na^+ in the medium was determined (Fig. 3.16). In the presence of residual amounts of Na^+ , only minute succinate uptake rates were observed. Increasing the Na^+ concentration caused an increase in the succinate uptake rate, reaching its maximal value of $174 \pm 21 \text{ nmol min}^{-1} (\text{mg cdm})^{-1}$. A K_m of $1.6 \pm 0.5 \mu\text{M}$ for Na^+ was derived upon fitting of these data according to the Michaelis-Menten equation.

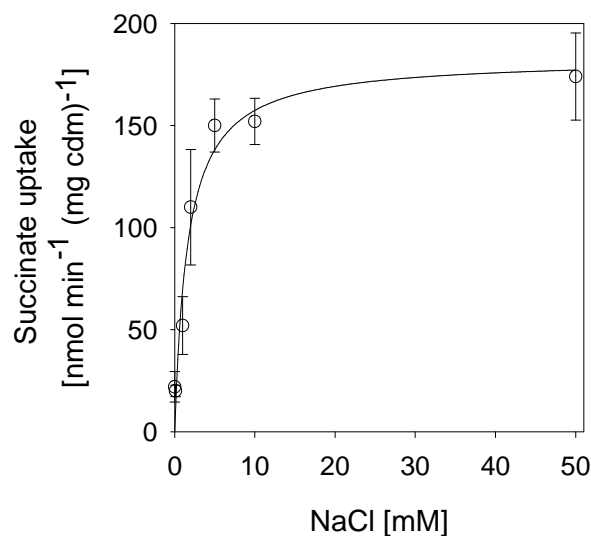


Fig. 3.16 Ion dependence of succinate uptake. Succinate uptake by *C. glutamicum* SSM was measured in dependence on the external NaCl concentration and measurements were performed at V_{\max} in the presence of 800 μM succinate. $n=3$.

The strong dependence of succinate uptake in the SSM mutant expressing *dccT* on Na^+ availability suggests that DccT is a Na^+ dependent secondary active dicarboxylate importer.

3.4.6 DccT also imports oxaloacetate

In order to get a first hint at which substrates DccT might accept besides the previously mentioned succinate, fumarate, and malate, competition assays were carried out. Therefore, the uptake of succinate was measured in the *C. glutamicum* pVWEx1-*dccT* strain in the presence of 100 μM succinate and the putative substrates were added in 100 fold excess. The chosen substrates were intermediates of the TCA, L-glutamate and L-aspartate as

further dicarboxylates and glyoxylate, L-lactate and pyruvate as monocarboxylates. As listed in table 3.8, only oxaloacetate reduced severely the succinate uptake ability of DccT. This result suggests that oxaloacetate might be accepted by DccT as well, but whether it is only bound by the protein and thus blocks the substrate binding site or whether it is actually translocated has to be proven directly.

Table 3.8 Succinate uptake by *C. glutamicum* pVWEx1-*dccT* strain upon the addition of several organic acids. Competing substrates were added in 100 fold excess in the presence of 100 μ M succinate. Uptake rates are given as percentage of the control rate of 38 ± 2 nmol min⁻¹ (mg cdm)⁻¹. n=3.

Substrate added	Succinate uptake [%]
Control	100 \pm 4
Citrate	125 \pm 2
Isocitrate	127 \pm 9
2-Oxoglutarate	91 \pm 2
Fumarate	1 \pm 2
L-Malate	32 \pm 0
Oxaloacetate	2 \pm 1
Glyoxylate	82 \pm 1
Pyruvate	123 \pm 8
L-Lactate	89 \pm 6
L-Glutamate	119 \pm 10
L-Aspartate	116 \pm 4

To demonstrate DccT uptake ability of oxaloacetate, growth experiments were carried out on oxaloacetate as sole carbon and energy source. It had to be considered, that oxaloacetate spontaneously decomposes to pyruvate (Nisonoff *et al.*, 1953), which can be utilized by *C. glutamicum*. In order to discriminate between growth on pyruvate and oxaloacetate, the experiments were carried out with the $\Delta mctC$ pVWEx1-*dccT* strain, lacking the pyruvate uptake system and expressing the putative oxaloacetate importer.

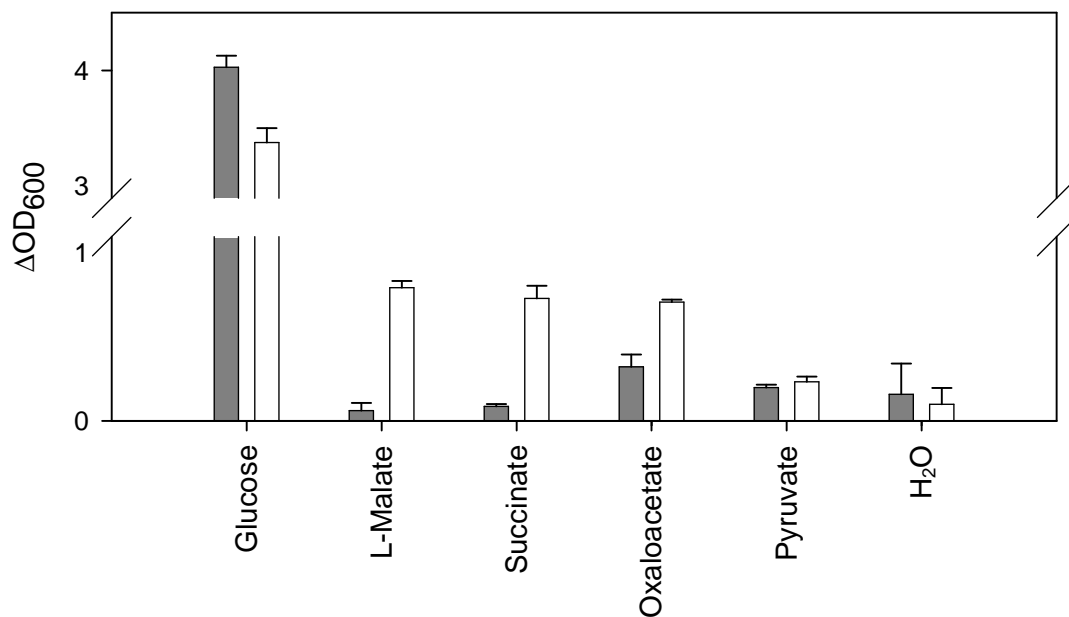


Fig. 3.17 Growth of *C. glutamicum* $\Delta mctC$ pVWEx1 (filled bars) and *C. glutamicum* $\Delta mctC$ pVWEx1-*dccT* (open bars) on 50 mM of each substrate. Growth was carried out in microtiter plates and the final OD₆₀₀ after 21 h incubation time is given. n=3.

As demonstrated by this experiment, the introduction of *dccT* caused growth of $\Delta mctC$ on dicarboxylates (Fig. 3.17). Growth on pyruvate was not distinguishable from residual growth in medium lacking a carbon source, demonstrating once more the indispensability of MctC for pyruvate uptake. Cells that did not express *dccT* showed also no growth on oxaloacetate. Finally, growth of *C. glutamicum* $\Delta mctC$ expressing *dccT* on oxaloacetate demonstrated that the presence of DccT is required for oxaloacetate utilization by *C. glutamicum* and thus confirmed that DccT indeed imports oxaloacetate.

Summing up the results for both dicarboxylate uptake systems, the presented data demonstrate that both DccT and DctA are dicarboxylate importers, the first being Na⁺ dependent, showing higher affinity towards succinate, fumarate, and L-malate than DctA but having a narrow substrate spectrum, since only oxaloacetate was identified as further substrate. DctA was shown to be H⁺ dependent and less affine towards those substrates, for which kinetic data were obtained. Moreover, it has a broad substrate spectrum accepting a variety of structurally different components.

3.5 Carboxylate export

Besides import, also the export of carboxylates represents an important factor in carboxylate metabolism. The understanding of export processes is of pivotal importance for correct estimation of substrate fluxes. Moreover, it is advantageous for biotechnological applications, either enhancing substrate efflux or on the contrary limit excretion of an undesired by-product and thus carbon loss. Therefore, the second part of the work focused on the setting of substrate exporting conditions and the identification of the corresponding export system.

3.5.1 Lactate, succinate, and acetate are exported at microaerobic conditions

The excretion of the organic acids lactate, succinate and acetate under oxygen deprived conditions has been reported for many cells and also for the strain *C. glutamicum* R (Inui *et al.*, 2004). Whether this is true for the strain used in this study was tested in the following experiment. *C. glutamicum* was grown to mid-exponential phase to OD₆₀₀ of approx. 10 and concentrated by centrifugation, reaching thus quickly higher cell densities. From here on, the culture was incubated at 30°C in a sealed tube and samples were taken without opening through the rubber seal. A quick consumption of oxygen under these conditions was reported by Inui *et al.* (2004). As depicted in Fig. 3.18, *C. glutamicum* excreted substantial amounts of lactate at an accumulation rate of $2.5 \pm 0.2 \text{ mM h}^{-1} (\text{mg cdm})^{-1}$ and minor amounts of succinate and acetate at $0.26 \pm 0.02 \text{ mM h}^{-1} (\text{mg cdm})^{-1}$ and $0.04 \pm 0.01 \text{ mM h}^{-1} (\text{mg cdm})^{-1}$, respectively. In further prolonged experiments, up to 140 mM lactate were observed in the medium after 4 h of incubation time (data not shown).

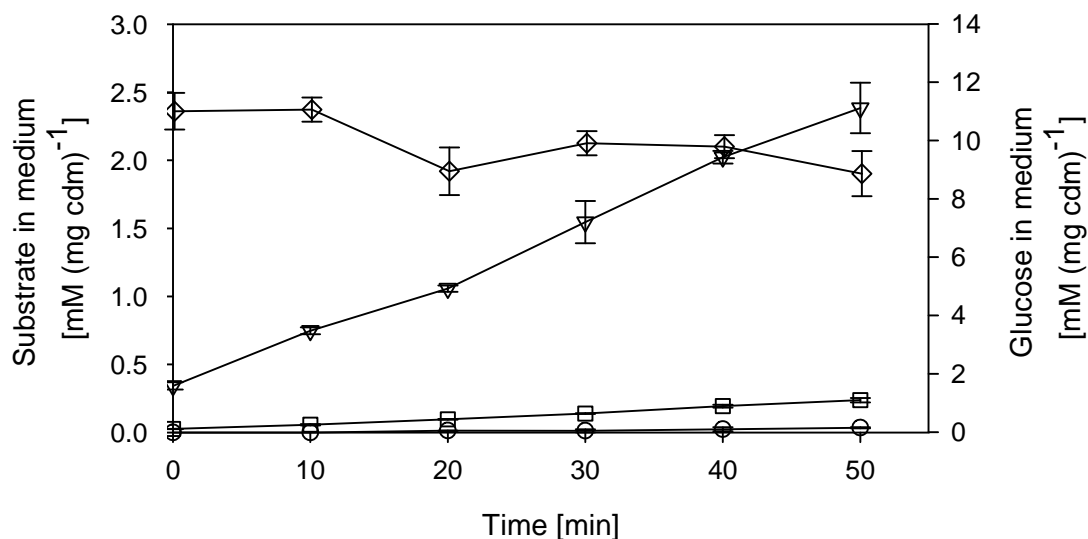


Fig. 3.18 Accumulation of lactate (triangles), succinate (squares), acetate (circles) and the consumption of glucose (diamonds) after onset of microaerobic conditions. n=3.

Thus, *C. glutamicum* ATCC 13032 converts most of the glucose to lactate and excretes it into the medium, causing external lactate accumulation. As described in 3.3, L-lactate uptake measurements showed that the *C. glutamicum* membrane is likely to be impermeable for lactate under the tested conditions. Therefore, its export might be also mediated by a transporter, which needs to be identified in this organism.

3.5.2 Lactate exporter candidates

Although lactate export occurs in many different organisms, only 3 lactate export systems have been identified yet. Those were shown to catalyze lactate export in antiport with citrate as CitP from *L. mesenteroides*, or with malate as MaeN or MleP from *B. subtilis* and *L. lactis*, respectively (Marty-Teyssset *et al.*, 1995; Bandell *et al.*, 1998; Wei *et al.*, 2000). *C. glutamicum* excretes lactate without external addition of any substrate, indicating that an antiporter is unlikely the major lactate efflux system. However, parallel excretion of the countersubstrate and its subsequent re-uptake in exchange with lactate could not be excluded and was therefore also considered. Comparing the amino acid sequence of the above mentioned proteins with the *C. glutamicum* proteome, only a homologous protein showing 84% identity to CitP was found. This was Cgl0067, being annotated as CitM, which was proposed to be the Mg^{2+} -citrate symporter (Polen *et al.*, 2007). This candidate was subsequently analyzed for lactate export ability by comparing lactate production of the wild type with a mutant with an interrupted *citM* gene, but no difference in the external accumulation rate was observed (Fig. 3.19A).

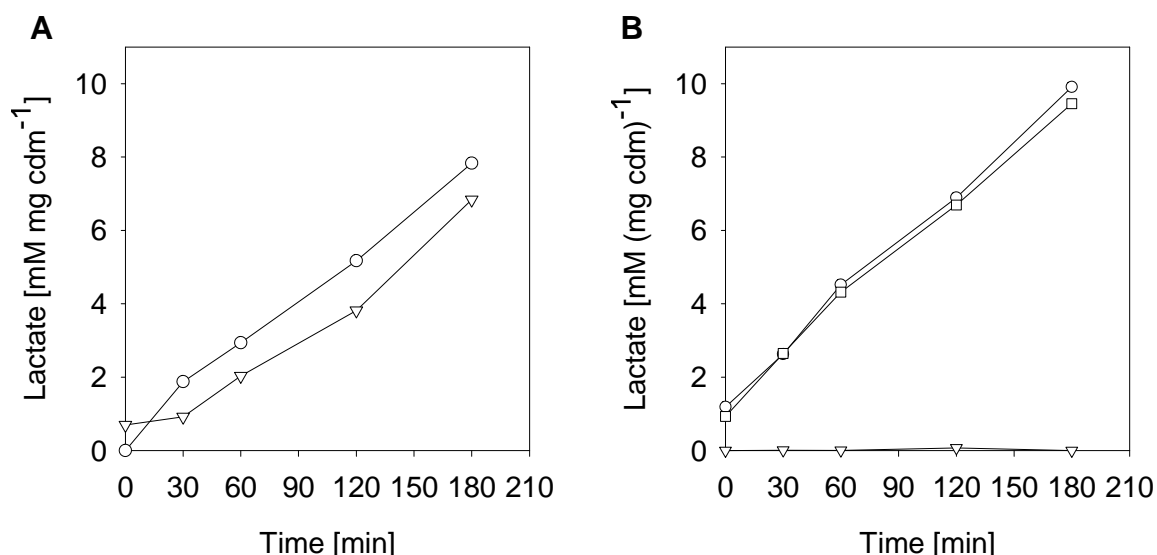


Fig. 3.19 Lactate accumulation by several lactate exporter candidates. The wild type is indicated by circles and the mutant *icitM* is indicated by triangles in (A), *ldhA* by triangles and *icgl2912* by squares in (B).

Another approach for identification of a putative lactate exporter candidate was the analysis of the genetic arrangement of *ldhA* encoding the lactate evolving lactate dehydrogenase. As

depicted in Fig. 3.20, downstream and in the divergent direction of *ldhA* is located *cgl2912* encoding a putative membrane protein with 5 transmembrane domains. Further putative transporter encoding genes were not found in direct vicinity of *ldhA*. The concomitant expression of *ldhA* and *cgl2912* was assumed due to the possibly shared regulatory elements in their promoter region, which would be expected if Cgl2912 was the lactate exporter. Thus, a mutant carrying an insertion in the transporter encoding gene as well as *ildhA* were tested for lactate export. As expected, *ildhA* strain excreted no lactate (Fig. 3.19B), demonstrating the presence of a singular lactate evolving enzyme at microaerobic conditions. However, the knock-out of the adjacent putative transporter encoding gene did not result in any significant difference in lactate efflux compared to the wild type. Thus, no lactate exporter candidate could be derived from operon structure analysis.

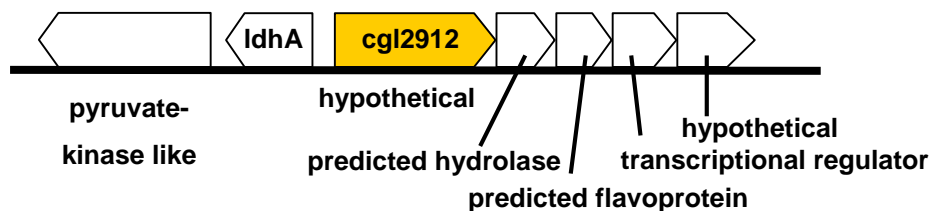


Fig. 3.20 Genetic arrangement of *ldhA* and adjacent genes. Genes encoding proteins with transmembrane domains are indicated by filled arrows.

This result indicates that *C. glutamicum* does not make use of concomitant expression of lactate converting and exporting proteins from an operon. This does not need to hold true for other organisms. Therefore, bacterial genomes were analyzed for genes encoding proteins with >3 transmembrane helices putatively transcribed in an operon with *ldhA*. Subsequently, sequence comparisons were performed in order to identify homologous proteins in *C. glutamicum*. A search in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database for *ldh* resulted in 259 bacterial genes. All adjacent genes coding for a protein with transmembrane domains were compared by Blast analysis with the *C. glutamicum* proteome. Genes showing high sequence similarity with an E-value of $\leq 10^{-11}$ are listed in table 3.9.

Results

Table 3.9 Genes putatively transcribed in an operon with *ldhA* in different bacteria and their respective homologue in *C. glutamicum* as determined according to the amino acid sequence of the derived protein.

Organism	Gene	Annotation	Homologue in <i>C. g.</i>	E-value
<i>Cytophaga hutchinsonii</i>	CHU_1629	magnesium transporter, MgtE	Cgl0224	1e ⁻³²
<i>Bordetella pertussis</i>	BP0380	putative sodium:sulfate symporter	Cgl0225	9e ⁻²³
<i>Ralstonia eutropha</i>	H16_A0667	sodium:sulfate symporter	Cgl0225	3e ⁻²⁵
	H16_A1680	predicted permease	Cgl0590	2e ⁻¹⁴
<i>Burkholderia mallei</i>	BMA10229_A2693	MFS transporter	Cgl0670	4e ⁻³⁸
<i>Sorangium cellulosum</i>	sce5246	hypothetical protein	Cgl1270	1e ⁻¹¹
<i>Bifidobacterium adolescentis</i>	BAD_1116	cation efflux system protein, CDF family	Cgl1281	3e ⁻³³
<i>Bordetella pertussis</i>	BP0380	putative sodium:sulfate symporter	Cgl2045	3e ⁻²⁰
<i>Lactobacillus plantarum</i>	lp_1102	divalent anion:Na ⁺ symporter, DASS family	Cgl2045	1e ⁻⁹⁶
<i>Lactobacillus acidophilus</i>	LBA0912	divalent anion:Na ⁺ symporter, DASS family	Cgl2045	3e ⁻⁹⁰
<i>Ralstonia eutropha</i>	H16_A0667	sodium:sulfate symporter	Cgl2045	8e ⁻²³
	H16_A1680	predicted permease	Cgl2211	3e ⁻¹⁸

In order to test the contribution of these putative transporters to lactate efflux in *C. glutamicum*, the corresponding genes were knocked-out and the created mutants assessed for lactate export abilities. As shown in Fig. 3.21, lactate export and glucose consumption were comparable in the wild type and the mutant strains with the exception of *icgl2211*. No lactate accumulation was observed in the medium of this strain and glucose consumption was also strongly reduced.

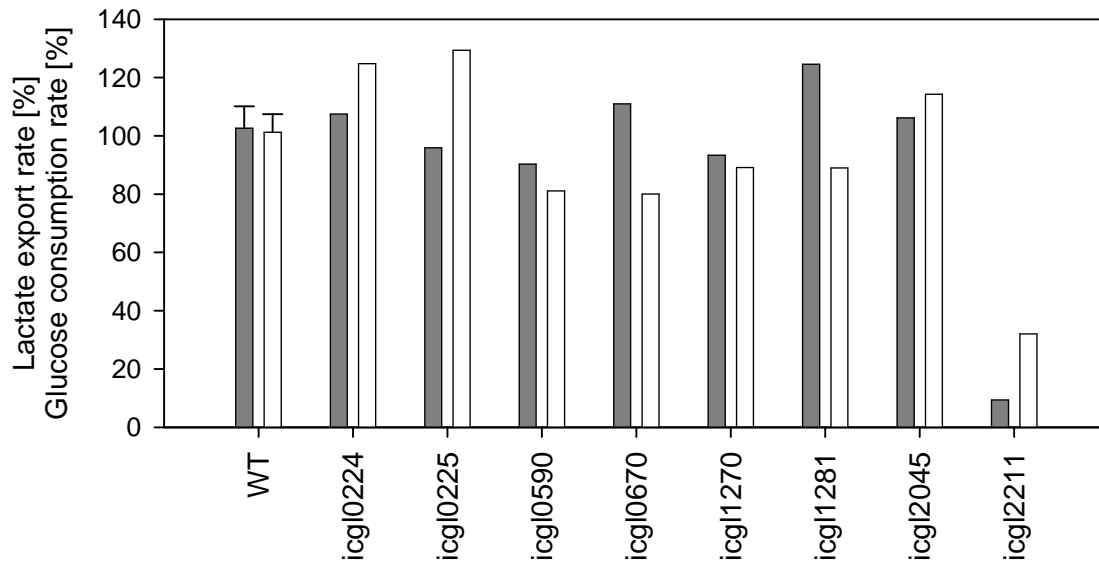
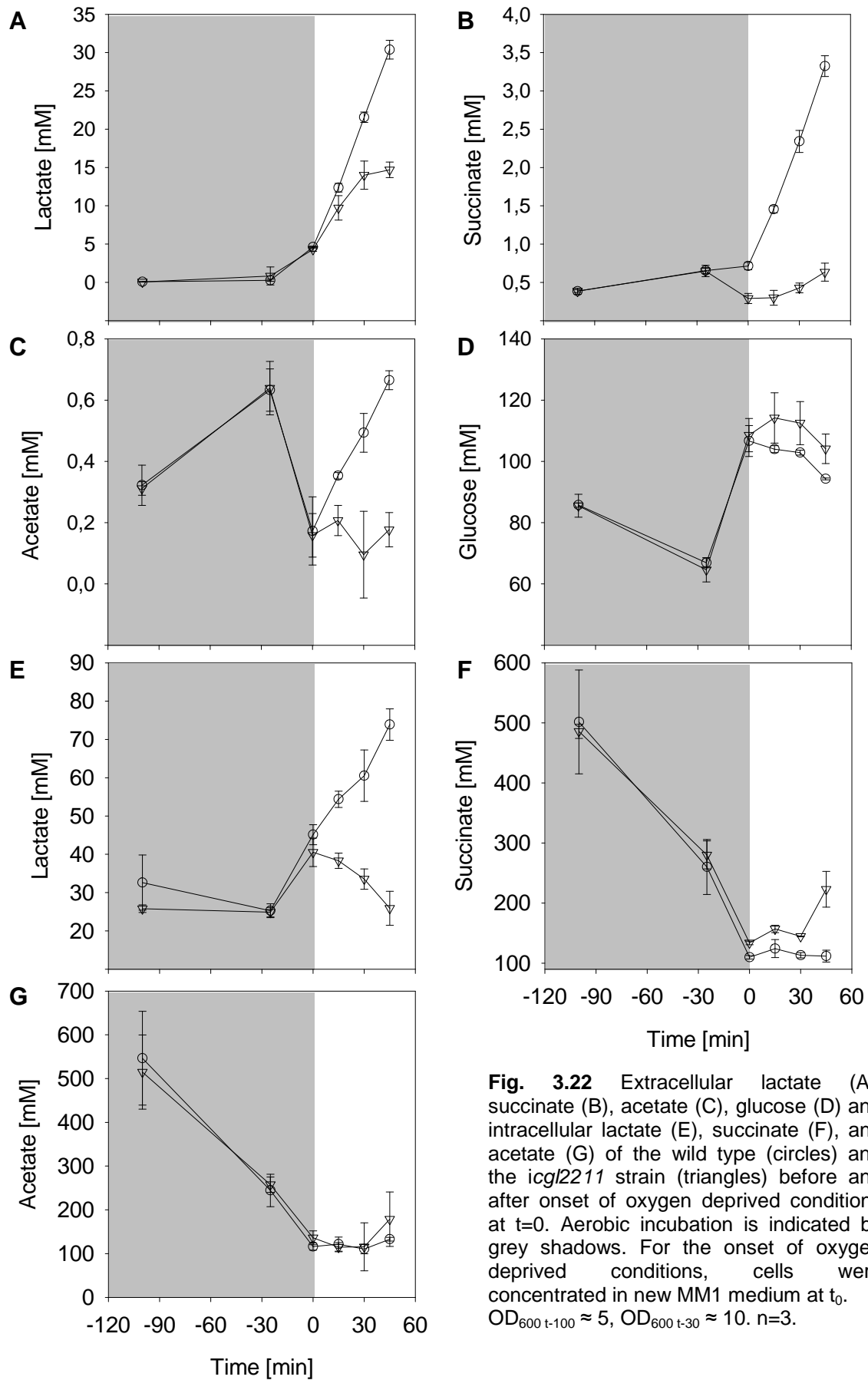


Fig. 3.21 Lactate export (filled bars) and glucose consumption (open bars) rates of several putative lactate exporter candidates. For mutant selection confer text. Wild type (WT) absolute lactate export rate was $4.0 \pm 0.3 \text{ mM h}^{-1} (\text{mg cdm})^{-1}$ and glucose consumption rate was $2.4 \pm 0.1 \text{ mM h}^{-1} (\text{mg cdm})^{-1}$, which were set as 100%. $n=3$ for WT.

In order to further investigate the role of Cgl2211 in lactate export, the strain was subjected to thorough analysis concerning both internal and external substrate concentrations. Besides lactate, the other fermentative end-products succinate and acetate were also monitored. Accumulation of a substrate would indicate an impaired export system. Moreover, substrate concentrations were determined also before onset of anaerobiosis at OD_{600} of approx. 5 and 10. As shown in Fig. 3.22, *icg12211* showed no difference in metabolite concentrations before onset of microaerobic conditions. Also growth of *icg12211* and the wild type was comparable (data not shown). After the beginning of oxygen deprivation conditions ($t=0$), lactate accumulated in the medium, but slower than in the wild type and its accumulation ceased after 30 min (Fig. 3.22A). Moreover, acetate and succinate accumulation were strongly reduced in this strain (Fig. 3.22B, C). Also less glucose was consumed (Fig. 3.22D). Internal values for acetate were comparable, lower for lactate and increased in the case of succinate (Fig. 3.22E-G). This experiment showed that although Cgl2211 is not involved in lactate export, its knock-out has dramatic consequences on microaerobic metabolism, changing the carboxylate pools in *C. glutamicum*. Whether this is due to impaired succinate export as might be assumed from the increasing internal succinate concentration and decreased succinate efflux or whether this phenotype is caused by any secondary effect remains to be tested in further experiments.



Since a rational selection of putative lactate exporters failed to indicate a lactate exporter candidate, a broad screening assay was undertaken. This approach required the fulfillment of two conditions in order to lead to a lactate exporter candidate. First, the deletion of the lactate exporter must not cause a severe growth phenotype under aerobic conditions, otherwise the creation of the mutant would be hard to accomplish. Second, *C. glutamicum* must have only one major lactate export system, otherwise the strain carrying the mutation in the lactate exporter will not be identified in the screen, if the presence of another exporter is sufficient to maintain lactate efflux at comparable rates.

In the first place, 90 site-directed mutants available at that time were assayed for lactate export. Due to the high number of strains, the standard lactate export assay was not applicable and had to be modified. One alteration was the determination of lactate in the medium, which was performed enzymatically in microtiter plates. Moreover, the experimental setup was modified. The strains were pre-cultivated in BHI complex medium in microtiter plates, transferred to a microtiter plate with MM1 medium, sealed with a gas-impermeable membrane and cultivated without shaking. After several time points, samples were taken and 10 μ l of the supernatant was assayed enzymatically for lactate production. A time-dependent increase in lactate was observed with discriminable lactate concentrations in the first 14 h of incubation time (Fig. 3.23A). However, a severe limitation of the test was the limited lactate concentration range that could be linearly correlated to NADH formation (Fig. 3.23B).

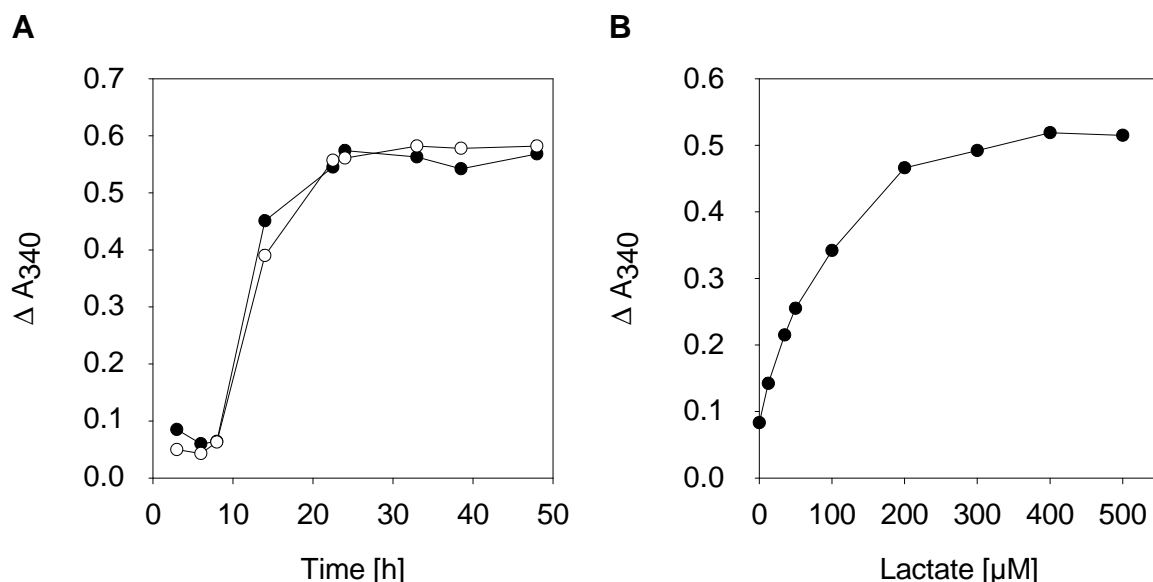


Fig 3.23 A) Lactate production of *C. glutamicum* in microtiter plates at microaerobic conditions. Data from two strains is given. B) Lactate calibration curve as measured in the Wallac multiplate reader at 340 nm. The change in A_{340} upon the production of NADH is plotted against lactate concentration.

With this method at hand, the site-directed mutant collection was assayed for lactate export. Cells were incubated for 14 h in sealed microtiter plates and the supernatant was tested for presence of lactate. As a control, the strain *icg/2089* lacking the pyruvate kinase encoding

gene was utilized. The data is presented in Fig. 3.24, where the NADH consumption was based on cellular dry matter. The mean value and the standard deviation were calculated over all strains.

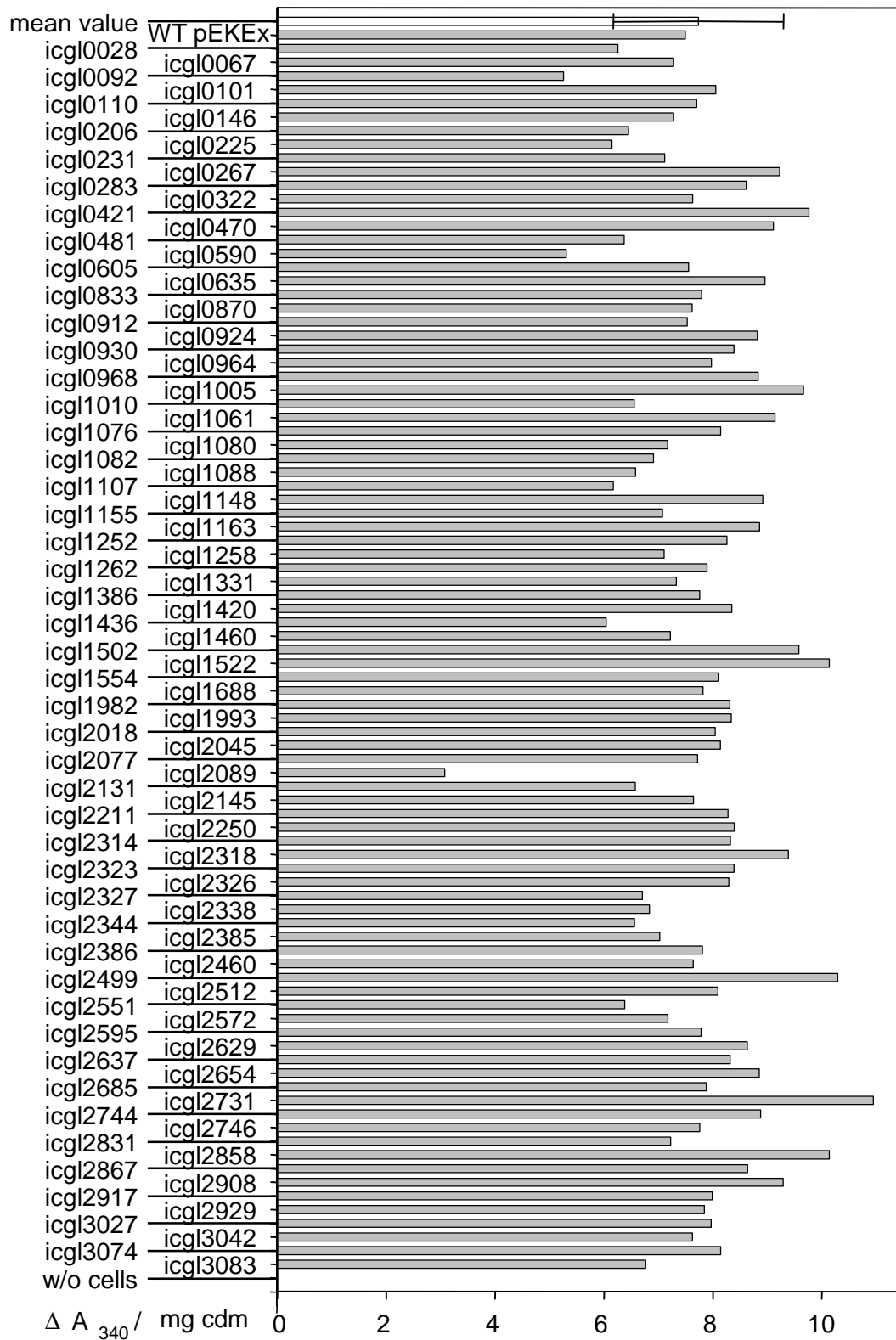


Fig. 3.24 Screen of the site-directed mutant collection for lactate export deficiency. The change in A_{340} is based on cdm and plotted for each strain (closed bars). As a control, the medium without cells and the medium of *C. glutamicum* pEKEx was assayed. The mean value (open bar) is calculated over all tested strains and the standard deviation is given.

Comparing the individual lactate production with the mean value, only the strains *icg10092*, *icg10590*, and *icg1436* showed a slightly reduced lactate production and *icg12089* lacking the pyruvate kinase showed a clear phenotype. The affected genes encode hypothetical membrane proteins (Cgl0092 and Cgl0590), and an NhaP like $\text{Na}^+\text{K}^+/\text{H}^+$ antiporter (Cgl1436). These mutants were thereupon assayed separately on large scale in sealed tubes, but no differences to wild type lactate efflux rates indicating an impaired lactate exporter were detectable (Fig. 3.25).

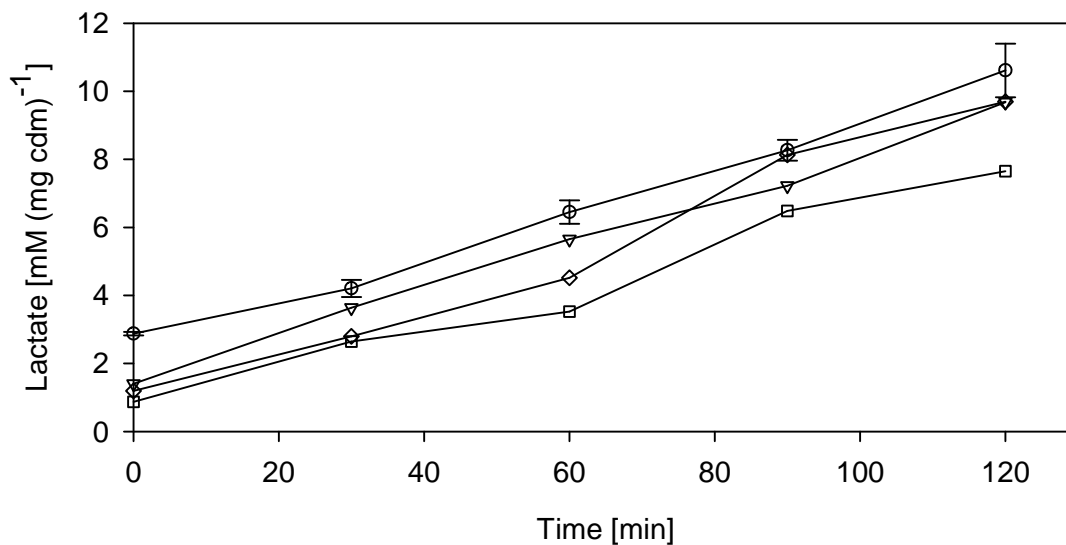


Fig. 3.25 Lactate efflux by selected mutants. WT is indicated by circles, *icg10092* by triangles, *icg10590* by squares and *icg1436* by diamonds. $n=3$ for wild type and $n=1$ for the mutant strains.

Thus, the assumption that one of the candidates was lacking the lactate exporter could not be proven. Nevertheless, the experiment indicated that screening for a lactate exporter in microtiter plates is possible. Taking into consideration that the mutant collection comprises only 40% of putative transporters, and 183 further membrane proteins might function as transporters as well, the chance of missing exactly the lactate exporter is given. Therefore, a larger mutant collection needed to be screened.

In 2006, Mormann *et al.*, published a successful screening of a transposon (Tn) mutant collection for enzymes involved in the histidine synthesis pathway. This collection comprises 10.080 clones, thus covering the genome 3 times. The authors showed random integration of the Tn element and calculated a probability of 97% for each gene to be inactivated. This mutant collection was kindly provided by J. Kalinowski and was screened for a lactate exporter as previously described for the site-directed mutant collection. Growth and lactate production as a change in A_{340} due to NADH consumption in the enzymatic assay was monitored.

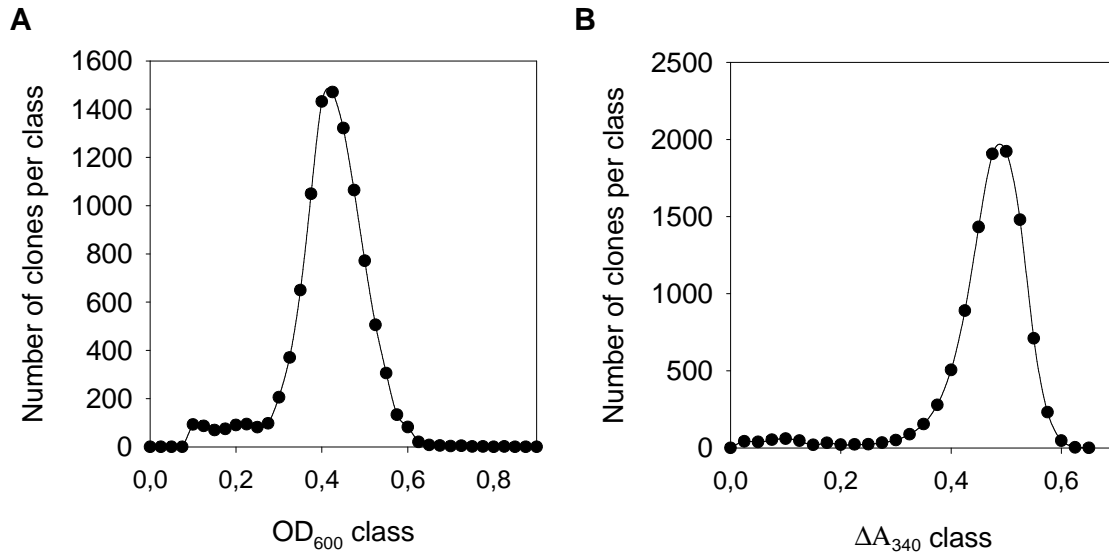


Fig. 3.26 A) Growth of the Tn mutant collection at microaerobic conditions. Final OD₆₀₀ values were grouped into classes of 0.025 units and the number of clones of a certain OD₆₀₀ class are plotted. B) Lactate production of the Tn mutants. Increase of NADH absorption was monitored at A₃₄₀, grouped into classes of 0.025 units and the number of clones of a certain A₃₄₀ class are plotted.

As depicted in Fig. 3.26A, the mutants showed evenly distributed growth at microaerobic conditions, reaching a mean OD₆₀₀ of 0.405 ± 0.088 . 77% of the mutants reached an OD₆₀₀ within the mean (μ) \pm standard deviation (σ) range and 11% showed a significantly lower growth than $\mu - \sigma$. As expected, the lactate production distribution represented here by ΔA_{340} was negatively skewed, indicating that most of the mutants produced lactate (Fig. 3.26B). A mean ΔA_{340} of 0.451 ± 0.085 was reached by 86% of the strains. However, a quantification of lactate amounts in the samples was not possible, since the value was out of the linear range of the lactate test (Fig. 3.23B). 8% of the Tn mutants showed the interesting phenotype with less lactate in the medium and were analyzed further on. Since this percentage corresponded to 944 strains, the screening has been performed again with these mutants to narrow down the number of candidates.

For those, the assay was modified since among the mutants showing a reduced lactate production were several strains growing bad at microaerobic conditions, falsifying the result. A bad growing culture utilizes less O₂, whereby more O₂ is present in the medium and consequently less lactate is produced than in a well growing one. As postulated for the screening assay, the lack of the lactate exporter should not have an impact on aerobic growth but no prediction can be made regarding growth at microaerobic conditions. To reduce this effect, the 944 re-screened strains were pre-cultivated aerobically for 16 h, reaching a higher OD₆₀₀ of 0.649 ± 0.345 than in the previous screen, but showing also a wider distribution regarding their growth. This time, a clear partition in two growth classes was observed (Fig. 3.27A): one reaching an OD₆₀₀ of 0.8 and the other one an OD₆₀₀ of 0.15, which corresponds to the OD₆₀₀ value of pure medium, indicating no growth. After

aerobic pre-growth, the strains were cultivated without shaking, allowing O_2 consumption and thus lactate production. As observed in the first screen, the left skewed distribution with ΔA_{340} of 0.406 ± 0.104 indicated lactate production by the majority (84%) of the mutants (Fig. 3.27B).

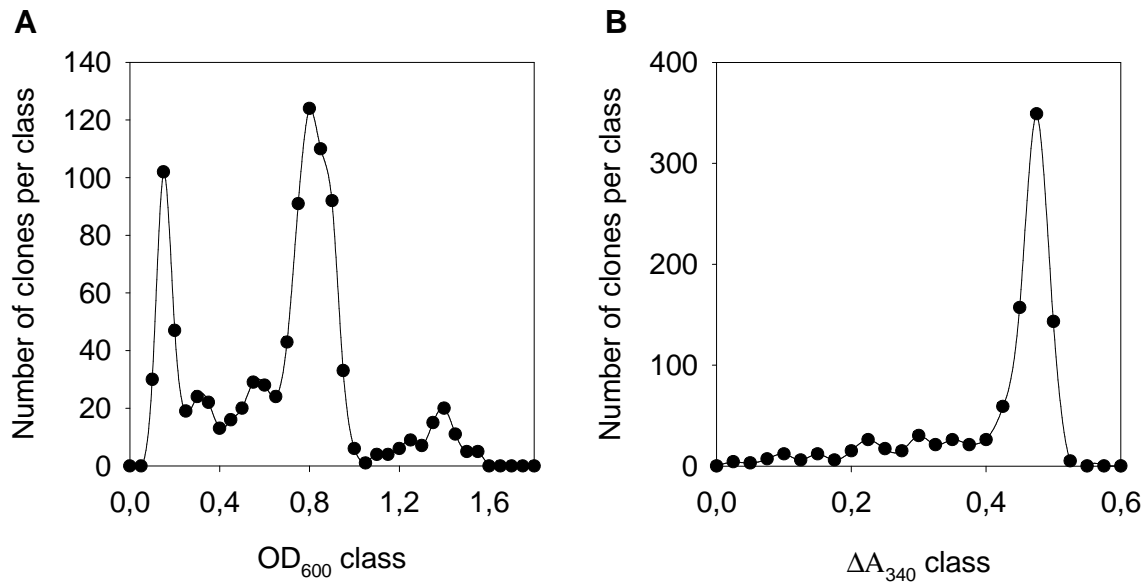


Fig. 3.27 A) Growth of the Tn mutant collection at aerobic conditions. B) Lactate production of the Tn mutants after 10 h of microaerobic incubation after aerobic pre-growth.

Strains that did not grow even under the initial aerobic conditions were neglected for further analyses. Combining these two criteria of growth and reduced lactate production (measured as $\mu\text{-}\sigma$), only 26 mutants were found to grow and to produce less lactate, being thus potential candidates with an impaired lactate export system. These strains were investigated in larger scale in sealed tubes and compared with the parent strain *C. glutamicum* RES 167. The determined lactate export rates are given in Fig. 3.28.

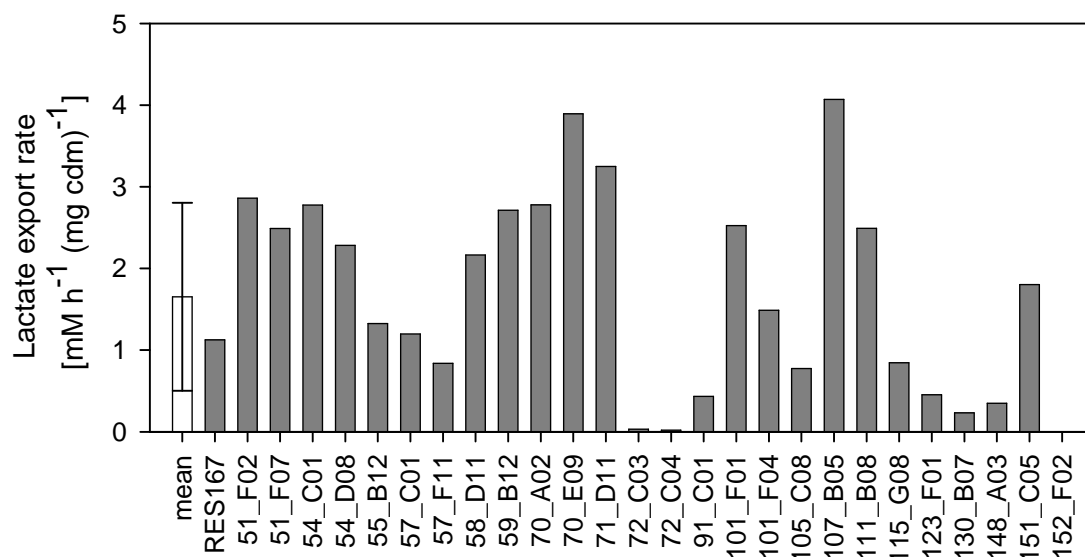


Fig. 3.28 Lactate export rate of selected TN mutants. For selection criteria confer text. Strain designation: nr. of MTP_{position} within the plate. The mean value is calculated over all 27 strains.

Thus, the strains 72_C03, 72_C04, 91_C01, 123_F01, 130_B07, 148_A03 and 152_F02 showed a significantly reduced lactate export rate. Therefore, these strains represented the best candidates for a strain lacking the lactate exporter. Moreover, growth experiments on minimal medium showed that the strains 91_C01, 123_F01 and 148_A03 grew slower on minimal medium under normal conditions than the *C. glutamicum* RES167 (data not shown). Therefore, these 3 strains were excluded from further work, since a growth phenotype on minimal medium at aerobic conditions due to the lack of the lactate exporter was not expected. Therefore, four transposon mutant strains were chosen for the determination of the transposon integration site, being considered as the best candidates for a knock-out in the lactate exporter encoding gene. For two strains, the Tn integration was not localized, and for the strains 72_C03 and 152_F02 the Tn integration was determined in phosphoglucosyltransferase encoding *pgi*. PGI is the decisive enzyme for the entry of glucose-6-P into glycolysis, converting glucose-6-P into fructose-6-P. Being deprived of this enzyme, *C. glutamicum* fuels glucose through the pentose phosphate pathway, but there is no obvious impact on lactate production, since both glycolysis and the pentose phosphate pathway lead to glyceraldehyde-3-P, the indirect precursor for pyruvate and thus lactate.

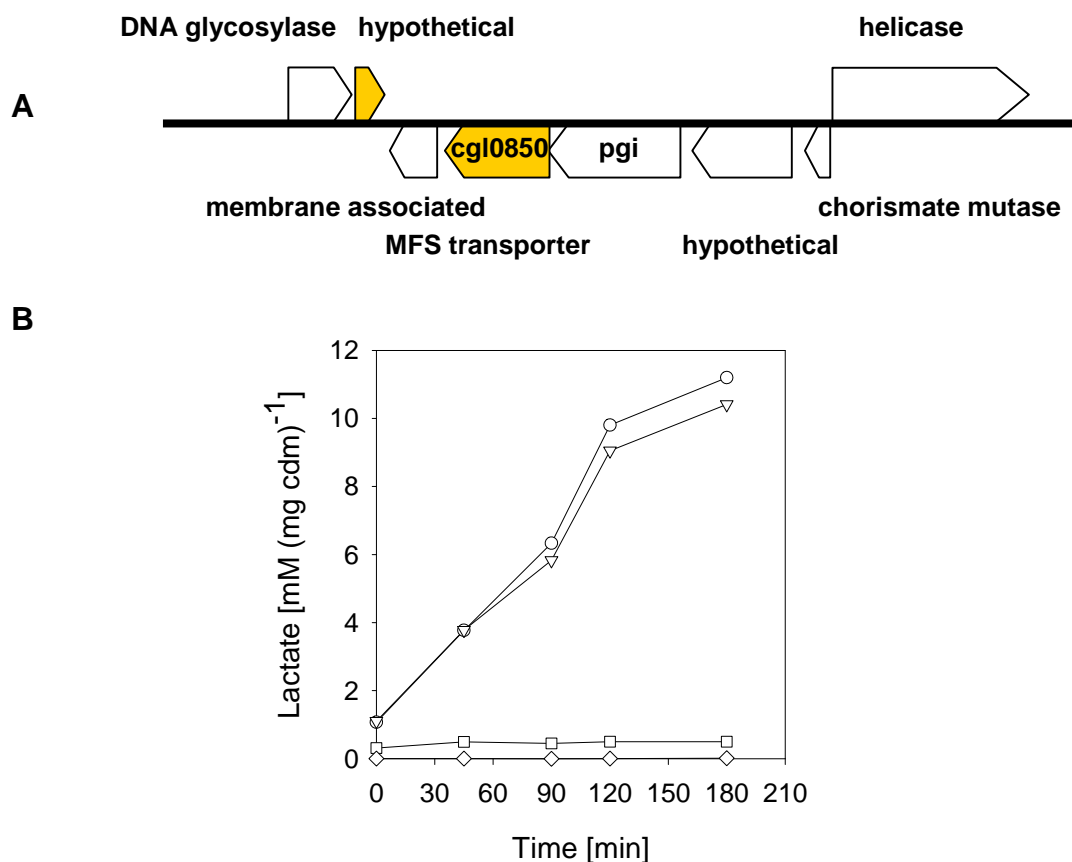


Fig. 3.29 A) Genetic arrangement of *pgi* and adjacent genes. Genes encoding proteins with transmembrane domains are indicated by filled arrows. B) Lactate export of site-directed knock-out mutants *icgl0850* encoding the MFS type transporter (triangles), Δpgi (diamonds), the Tn mutant 152_F02 (squares) and wild type as control (circles).

Analyzing the genetic localization of *pgi* for putative polar effects of the Tn insertion, an operon structure of *pgi* with an adjacent transporter encoding gene can be assumed due to their close localization (Fig. 3.29A). This gene is annotated as an MFS (major facilitator superfamily) type transporter. These catalyze solute uniport or transport with cation symport or antiport and accept a large variety of different substrates (Marin and Krämer, 2007). Whether the predicted MFS transporter plays a role in lactate efflux was tested by mutagenesis of the corresponding gene. Moreover, the Δpgi strain was assessed for lactate production in order to verify the phenotype of the Tn mutants. As shown in Fig. 3.29B, both the Tn strain 152_F02 and Δpgi strain produced no lactate in the assay. However, lactate accumulation of the *icgl0850* strain lacking the particular MFS type transporter in question was indistinguishable from lactate production by the wild type. Thus, the function of the MFS type transporter Cgl0850 as the major lactate efflux system can be excluded.

In order to complete this screening assay, the TN integration site for 72_C04 and 130_B07 needs to be determined. Whether the mutants have another gene than *pgi* interrupted by the Tn element and might thus indicate a potential lactate exporter needs to be accomplished in further experiments.

Summing up the results presented in this paragraph, several putative lactate exporter candidates were tested. All of them proved not to play a role in lactate efflux, and for one an indication for its role in succinate export is given. This strain needs to be analyzed further on, in particular by the construction and subsequent analysis of deletion and overexpression mutants and the determination of internal succinate concentration during prolonged incubation time. Transport assays with isolated and reconstituted carriers in liposomes would finally prove the participation of the corresponding transporter in substrate export. Furthermore, a large screening was carried out, resulting in the identification of phosphoglucosomerase as an essential enzyme for lactate production. However, no lactate exporter candidate was identified, neither by comparison with known or putative lactate exporters nor by the screening assay. The screening failure suggests the presence of more than one lactate export systems, whereby the individual candidates were missed in the assay. However, to prove this assumption, a thorough biochemical lactate export characterization is required.

3.5.3 Pyruvate is exported upon the inhibition of pyruvate dehydrogenase

Besides (micro)nutrient limitation as in the case of microaerobic conditions, a potent way to induce substrate efflux is the inhibition of enzymes converting the particular substrate. The inhibition might be permanent by the deletion of the corresponding gene or temporal by the application of an inhibitory agent. The efflux of the central metabolite pyruvate upon the deletion of the gene encoding the E1p subunit of the pyruvate dehydrogenase (PDH) has

been observed in *C. glutamicum* $\Delta aceE$ strain (Blombach *et al.*, 2007a). A temporal inhibition of the PDH results in the accumulation and efflux of pyruvate as well. For *Klebsiella pneumoniae*, the compound 1-aminoethylphosphinate (AEP) was demonstrated to be metabolized to acetylphosphinate, which caused an immediate inhibition of the PDH and the concomitant accumulation of pyruvate in the medium (Laber and Amrhein, 1987). The effect of AEP on *C. glutamicum* was tested in the following experiment. A *C. glutamicum* culture was grown in MM1 medium over night until the stationary phase, diluted in new MM1 with 200 mM glucose and 300 μ M 1-aminoethylphosphinate and incubated in a shaking flask. Growth as well as pyruvate production was monitored and depicted in Fig. 3.30.

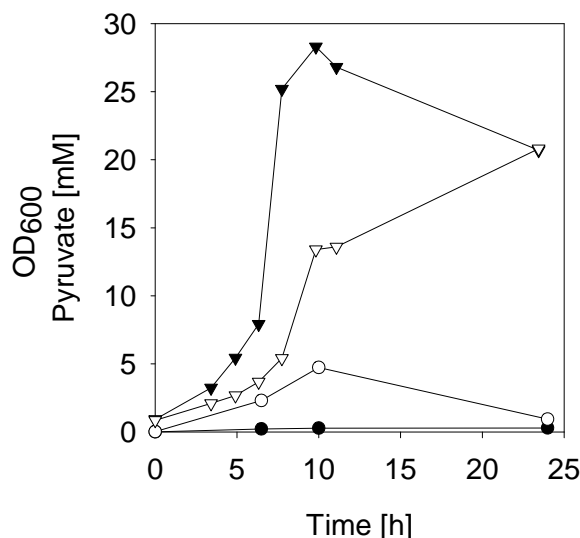


Fig. 3.30 Growth (triangles) and pyruvate production (circles) of *C. glutamicum*. The culture was cultivated in the presence (open symbols) or absence (closed symbols) of 300 μ M 1-aminoethylphosphinate.

Similar to *K. pneumoniae*, the application of AEP on *C. glutamicum* led to a growth retardation but final optical densities were comparable. Moreover, pyruvate was excreted into the medium where up to 5 mM pyruvate were detectable, but were re-utilized in the course of the experiment. Without the addition of AEP, only minor amounts of pyruvate were detected. Thus, the conditions for induced pyruvate export were set.

3.5.4 Pyruvate exporter candidates

Studies on pyruvate import in *C. glutamicum* have shown that its membrane is impermeable for this substrate (conf. 3.2.1). This observation suggested also a transporter mediated pyruvate efflux in *C. glutamicum*. To prove this assumption, as well as to investigate, whether the export system is constitutively expressed or induced upon pyruvate exporting conditions, the pyruvate export assay was carried out in the presence of the translation

inhibitor chloramphenicol. Moreover, the *C. glutamicum* $\Delta mctC$ strain was assessed to exclude re-uptake of pyruvate.

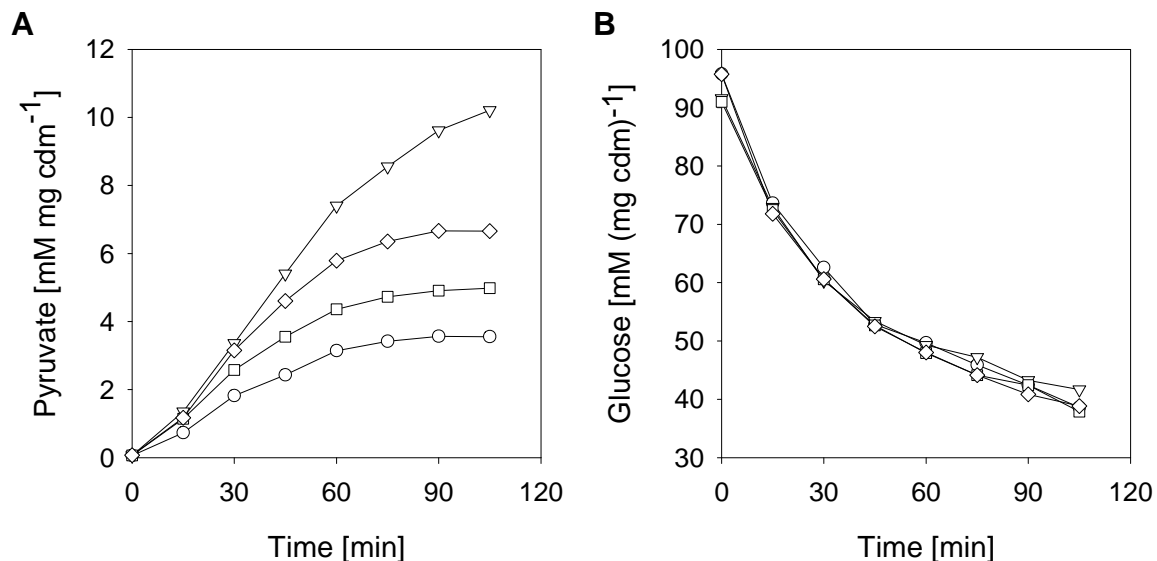


Fig. 3.31 A) Pyruvate accumulation in the medium and B) glucose consumption by *C. glutamicum* $\Delta mctC$ in the absence (triangles) or presence of chloramphenicol, which was added before (circles), 15 min (squares), and 30 min (diamonds) after the addition of AEP. $n=1$.

As depicted in Fig. 3.31, pyruvate export was markedly reduced upon the addition of chloramphenicol and ceased completely after 1h. At the same time, glucose consumption remained constant irrespective of chloramphenicol presence indicating functional metabolism. This result suggests that the exporter is already present before the application of AEP but requires constitutive synthesis to maintain pyruvate efflux. Whether the internal pyruvate concentration actually increased remains to be seen in further experiments.

Regarding the absent pyruvate efflux during normal cultivation (Fig. 3.30) and the requirement of its constitutive synthesis upon pyruvate efflux induction (Fig. 3.31A), an induced transcription rate of the exporter encoding gene was likely. Thus, a microarray analysis of differential gene transcription in order to find a pyruvate exporter candidate seemed appropriate. The experiment was performed with *C. glutamicum* wild type to exclude side-effects caused by the deletion of the pyruvate importer encoding *mctC*. In contrast to the previously described experiments, pyruvate exporting conditions were set on an exponentially growing culture to minimize the number of differentially expressed genes due to the entrance into the lag phase. The effect of AEP on exponentially growing cells was tested in preceding experiments and was comparable to the effect on cells from stationary phase, albeit less pyruvate was produced by the cells under these conditions (data not shown).

Table 3.10 Selected induced genes upon the induction of pyruvate production as determined in the microarray. Genes of known function and putative transporter encoding genes (highlighted) are listed. Transcript ratio before and after induction is given. For complete list confer table 7.4.

CDS	gene	gene product or deduced function	ratio
<i>cgI0248</i>	<i>leuA</i>	2-Isopropylmalate synthase	10.6
<i>cgI0708</i>	<i>dtbR1</i>	Acetyl/propionyl-CoA carboxylase, beta chain	2.8
<i>cgI0679</i>		Putative membrane protein	2.4
<i>cgI2004</i>		Putative secreted or membrane protein	2.4
<i>cgI1100</i>	<i>mshB</i>	Putative N-acetyl-1-D-myo-inositol-2-amino-2-deoxy-alpha-D-glucopyranoside deacetylase	2.3
<i>cgI0810</i>		ABC-type putative iron-siderophore transporter, substrate-binding lipoprotein	2.2
<i>cgI2911</i>	<i>ldh</i>	L-Lactate dehydrogenase	2.1
<i>cgI2227</i>	<i>hmuO</i>	Heme oxygenase (decyclizing)	2.0
<i>cgI0041</i>	<i>pknB</i>	Serine/threonine protein kinase	2.0
<i>cgI2610</i>	<i>pqo</i>	Pyruvate:quinone oxidoreductase	2.0
<i>cgI2026</i>	<i>rpsB</i>	30S ribosomal protein S2	1.9
<i>cgI0982</i>	<i>ripA</i>	Putative transcriptional regulator, AraC-family	1.9
<i>cgI0709</i>	<i>birA</i>	Bifunctional biotin-[acetyl-CoA-carboxylase] synthetase/biotin operon repressor	1.9
<i>cgI1259</i>	<i>gatB</i>	Glutamyl-tRNA (Gln) amidotransferase, subunit B	1.9
<i>cgI2872</i>	<i>fadD2</i>	Putative long-chain-fatty-acid-CoA ligase	1.8
<i>cgI2532</i>	<i>nrdH</i>	Conserved hypothetical protein, glutaredoxin-like protein NrdH	1.8
<i>cgI0525</i>	<i>dkg</i>	Putative aldo/keto reductase, related to diketogulonate reductase	1.8
<i>cgI2248</i>	<i>aceE</i>	Pyruvate dehydrogenase (acetyl-transferring)	1.7
<i>cgI2390</i>	<i>pcaI</i>	Putative 3-oxoadipate CoA-transferase	1.7
<i>cgI0343</i>	<i>cmT1</i>	Trehalose corynomycolyl transferase	1.7
<i>cgI0254</i>	<i>sigC</i>	RNA polymerase sigma factor, ECF-family	1.7
<i>cgI2942</i>		Putative membrane protein	1.7
<i>cgI2942</i>		Putative membrane protein	1.7
<i>cgI0086</i>	<i>ureC</i>	Urease alpha subunit	1.7
<i>cgI2927</i>	<i>sod</i>	Superoxide dismutase	1.7
<i>cgI2005</i>		Putative secreted or membrane protein	1.7
<i>cgI1223</i>	<i>ssuA</i>	ABC-type aliphatic sulfonate transporter, substrate-binding lipoprotein	1.6
<i>cgI1524</i>	<i>tetB</i>	ABC-type multidrug transport system, ATPase and permease subunit	1.6
<i>cgI0768</i>	<i>whcE</i>	Transcriptional regulator, WhiB-family	1.6
<i>cgI2301</i>	<i>dcp</i>	Peptidyl-dipeptidase	1.6
<i>cgI1395</i>	<i>argJ</i>	Glutamate N-acetyltransferase	1.6
<i>cgI2531</i>	<i>nrdI</i>	Conserved hypothetical protein, flavodoxin-like protein NrdI	1.6
<i>cgI1086</i>	<i>tpx</i>	Thiol peroxidase	1.6
<i>cgI1687</i>		ABC-type transporter, ATPase subunit	1.5
<i>cgI2770</i>	<i>fda</i>	Fructose-bisphosphate aldolase	1.5
<i>cgI1018</i>	<i>ssuD2</i>	FMNH ₂ -dependent aliphatic sulfonate monooxygenase	1.5
<i>cgI1163</i>	<i>putP</i>	Putative Na⁺/proline symporter, solute:sodium symporter (SSS) family	1.5
<i>cgI2411</i>	<i>clpP2</i>	Endopeptidase Clp, proteolytic subunit	1.5

For the microarray, samples from 2 biological replicates were taken before and 10 min after the addition of AEP. Of the approx. 3.300 annotated genes, only 1075 were detected in the experiment. The majority did not pass the quality control settings of the microarray evaluation or were not detected at all. Of the induced genes, only 106 showed an increased transcription and 103 showed a reduced transcription by a factor of ≥ 1.5 and ≤ 0.7 , respectively (table 7.4). For most of these genes, the function has not been proposed yet. Those with an assigned function and putative transporter encoding ones are listed in table

3.10. Among those, 9 are annotated as transporter encoding genes. 4 gene products are annotated as ABC-type transporters, one as a secondary active transporter of the SSS-type family (PutP) and 4 as hypothetical membrane proteins with 2-3 predicted transmembrane helices. The number of potential pyruvate exporters was further limited regarding the fact that 2 of the ABC-type transporters were predicted to have a periplasmic binding subunit, thus being rather import systems and Cgl2004 and Cgl2005 being rather short proteins of 116 and 120 amino acids constituting 2 transmembrane helices each. The corresponding genes are separated by 57 bp, suggesting the option that these two actually comprise one protein. PutP was previously described as a Na^+ dependent proline importer (Peter *et al.*, 1997), being also rejected as a putative pyruvate exporter. Thus, the number of pyruvate exporter candidates indicated by the microarray assay was restricted to 5: Cgl0679, Cgl1524, Cgl1687, Cgl2004-2005 and Cgl2942.

The selected pyruvate exporter candidates were subsequently knocked-out and tested for their pyruvate export ability. Instead of *cgl1687* encoding the ATPase component, the adjacent *cgl1688* encoding the permease component of the ABC-transport system was knocked-out to ensure loss of transport capability of this system. In the case of Cgl2004-2005, only *cgl2004* was knocked-out. External pyruvate accumulation was monitored in the early lag and in the exponential growth phase, which corresponded to the conditions tested in the microarray. *cgl2942* knock-out was not accomplished at that time but was included later on in the test presented in Fig. 3.34.

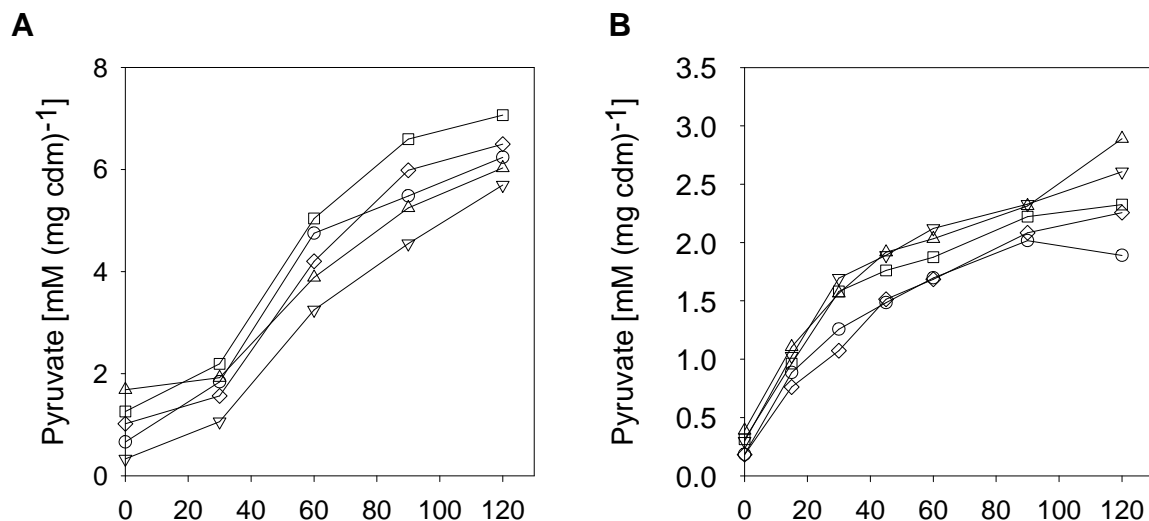


Fig. 3.32 Pyruvate export of *C. glutamicum* strains with a knocked-out gene for a putative pyruvate exporter as monitored in the lag (A) or exponential (B) growth phase. Pyruvate accumulation in the medium of *icgl0679* (triangles), *icgl1524* (squares), *icgl1688* (diamonds), *icgl2004* (upward triangles), and the wild type (circles) is depicted.

As shown in Fig. 3.32, none of the pyruvate exporter candidates, which were directly derived from microarray data, showed altered pyruvate accumulation in the medium. None of these proteins was essential for pyruvate export under the tested conditions.

As mentioned before, only a fraction of all annotated corynebacterial genes was detected on the DNA chip. Therefore, operon structures might have been represented only by the detected gene and the concomitantly transcribed ones might have been missed due to technical and not biological reasons. Regarding the genetic organization of genes identified in the microarray, several might be transcribed in an operon with a putatively transporter encoding one or from a promoter region with same regulator binding sites (Fig. 3.33).

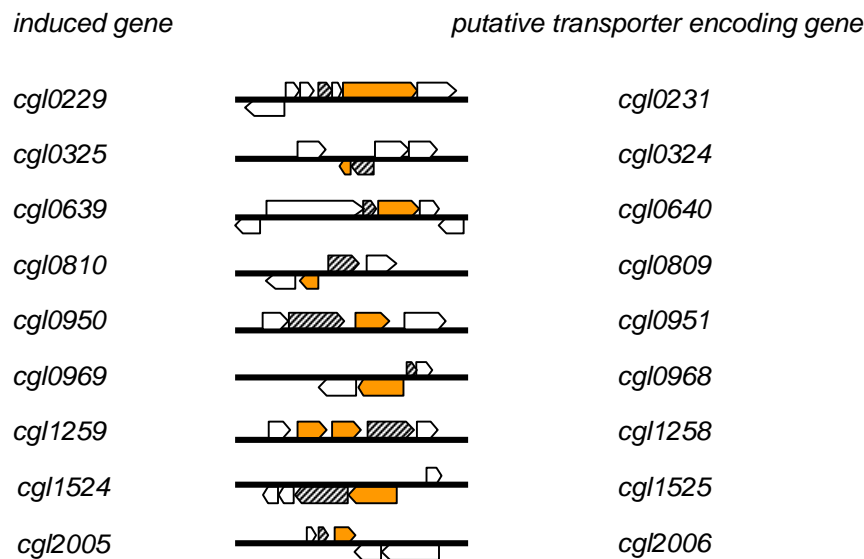


Fig. 3.33 Genetic organization of selected genes being induced under pyruvate producing conditions. Induced genes are represented by striped arrows. Filled arrows indicate putative transporter encoding genes. Open arrows represent adjacent genes coding for non-membrane proteins.

Thus, the putative membrane protein encoding genes *cgl0231*, *cgl0324*, *cgl0640*, *cgl0809*, *cgl0951*, *cgl0968*, *cgl1258*, *cgl1525*, and *cgl2006* were found in the neighborhood of induced genes and due to the expected co-transcription they were chosen as further candidates. Additionally, the remaining direct microarray candidate *cgl2942* was disrupted and the strain was tested as well. Furthermore, the two Cpa proteins Cgl0267 (CpaA) and Cgl2731 (CpaB) were chosen as candidates due to the observation of metabolite efflux via these systems (Ito *et al.*, 1999). Finally, the MFS type transporter Cgl2917 is encoded by a gene adjacent to the pyruvate evolving lactate dehydrogenase encoding gene *lldD* and was thus chosen due to its possible valve function during pyruvate overflow upon lactate oxidation. Up to now, insertions were not successfully introduced into *cgl0809* and *cgl2006*, but the other candidates were tested in an initial assay for their pyruvate export ability.

Initial tests regarding the pyruvate production of the tested strains showed that *icgl0231*, *icgl0267*, *icgl0324* and *icgl2917* produced less pyruvate but - with the exception of *icgl0231* - comparable glucose consumption as the wild type (data not shown). However, further data on these mutants is needed to validate the function of the missing protein as a pyruvate

exporter. In particular, data on internal pyruvate concentrations would indeed indicate the involvement of a certain protein in pyruvate export, if internal substrate accumulation is observed. Complementation and overexpression studies and eventually transport assays with the isolated and reconstituted transporter will be required in order to prove the function of a certain protein as the pyruvate exporter.

Summing up the data obtained in studies concerning pyruvate export, the conditions to trigger pyruvate efflux were set for *C. glutamicum*. Indications for the presence of a pyruvate export system were given. 4 pyruvate exporter candidates were identified, but need to be confirmed in further experiments.

4 Discussion

The metabolism of carboxylates encompasses not only their anabolism and catabolism within the cell, but also their uptake and excretion plays an important role in cellular fluxes. The understanding of carboxylate metabolism is of particular interest for fundamental research, approaching the goal of complete understanding of metabolic pathways, and also for biotechnological applications, enabling improvement of production processes by increasing the yield and reducing the production costs. The availability of cheaper carboxylates would foster their utilization for further processes as for example the production of degradable polymers, which cannot be realized yet on large scale due to the high costs of chemical organic acid synthesis among other reasons. With this perspective, research on a biotechnologically established microorganism as *C. glutamicum* is of special importance.

In this study, the importers for the monocarboxylates acetate, propionate, pyruvate, and glyoxylate were identified and an L-lactate importer suggested. Two dicarboxylate importers were identified which were shown to be decisive for the uptake of succinate, fumarate, L-malate, and oxaloacetate. Moreover, the import of citrate, isocitrate, 2-oxoglutarate, and L-aspartate was suggested to proceed via one of these systems.

Phenotypic analysis of site-directed mutants indicated a succinate exporter candidate. A broad screening assay led to the identification of phosphoglucosomerase as an essential enzyme for lactate production during microaerobic conditions. However, no lactate exporter candidate could be assigned, suggesting the presence of several lactate export systems in *C. glutamicum*. Finally, transcriptome analysis during pyruvate-producing conditions showed differential expression of genes encoding pyruvate utilizing enzymes. This approach as well as phenotypic characterization of site-directed mutants helped to identify putative pyruvate exporter candidates.

4.1 Carboxylate import

The utilization of carboxylates depends severely on the ability of the organism to import them. As they are charged molecules and cannot freely pass the cytoplasmic membrane, their import requires the presence of an active uptake system. Several monocarboxylate transporters were identified or postulated in the last couple of years. The eukaryotic Jen1P from *Saccharomyces cerevisiae* was reported to import lactate, acetate, pyruvate and propionate in a proton dependent manner (Soares-Silva *et al.*, 2003) and several MCT transporters were identified in mammals, yeast and archaeobacteria (Price *et al.*, 1998; Halestrap and Price, 1999). However, bacterial monocarboxylate transporters do not show similarity to MCTs. Biochemically characterized secondary active transporters are members of the SSS type transporter family such as MctP and ActP or of the Lactate Permease (LctP)

family (Hosie *et al.*, 2002; Gimenez *et al.*, 2003; Núñez *et al.*, 2002). Dicarboxylates are imported via distinct secondary active transporters of several classes (Janausch *et al.*, 2002). At the beginning of this work, no carboxylate transporters were known for *C. glutamicum*, although the transport of malate, acetate, succinate, lactate, and pyruvate has been observed (Menkel *et al.*, 1988; Ebbighausen *et al.*, 1991a,b; Inui *et al.*, 2004; Blombach *et al.*, 2007a). Also the utilization of several carboxylates as carbon sources suggested the presence of the corresponding transport system (Cocaign *et al.*, 1993; Wendisch *et al.*, 2000; Claes *et al.*, 2002; Netzer *et al.*, 2004). In the following, three new carboxylate transporters of *C. glutamicum* will be described, which were identified in the course of this work.

4.1.1 The monocarboxylate importer MctC is a member of a new subclass of SSS-type transporters

Phenotypic analysis of site-directed mutants suggested that Cgl0833 (MctC) is a pyruvate importer (Ballan, 2007). Subsequent analysis of the $\Delta mctC$ strain performed here showed its severely reduced pyruvate, acetate, and propionate import abilities, which indicated the participation of MctC in the import of these substrates.

Regarding the kinetic data of MctC, the $K_{0.5}$ determined in this study for acetate (32 μM) corresponds to the previously published K_m of 50 μM (Ebbighausen *et al.*, 1991a) and is in the range of the $K_{0.5}$ obtained for propionate uptake (9 μM). The affinity for pyruvate, whose uptake completely relies on the presence of MctC, is by far lower than for the membrane permeable substrates acetate and propionate (249 vs. 32 and 9 μM). This might reflect the fact that acetate and propionate are frequently found in soil as fermentative end-products during short oxygen supply (Rothfuss and Conrad, 1993) but the presence of pyruvate has not been reported yet.

Additional MctC substrates were not identified in this study, which is in agreement with the rather narrow substrate spectrum of the other previously described monocarboxylate importers. For ActP from *E. coli* only acetate and propionate were shown to be translocated (Gimenez *et al.*, 2003) and LldP and GlcA accepted only lactate and glycolate (Núñez *et al.*, 2002). Another biochemically characterized transporter is MctP from *R. leguminosarum*, which is rather outstanding, since it was shown to mediate lactate, pyruvate and alanine transport and uptake inhibition by propionate, butyrate and α -hydroxybutyrate indicated binding of these substrates as well (Hosie *et al.*, 2002).

According to the IUBMB classification of transporters (Busch and Saier, 2004), MctC, ActP and MctP are members of the SSS-type (solute:sodium symport) transporter family, although no indication for Na^+ dependence of solute transport was found for neither of them. For MctC, data proving its dependence on the proton membrane potential was presented (Fig. 3.4) and proton dependent acetate uptake has also been described in *C. glutamicum*

by Ebbighausen *et al.* (1991a). Furthermore, the Hill coefficient >2 for the C_3 substrates pyruvate and propionate indicated cooperative binding of the substrate. Although this was not observed for the C_2 substrate acetate, further detailed analyses regarding the H^+ :substrate stoichiometry might shed light on this problem. However, for the other SSS type transporter PutP of *C. glutamicum* Na^+ dependence of proline import was demonstrated (Peter *et al.*, 1997). Comparing the MctC sequence with other bacterial SSS-type transporters, three different transporter clusters within the SSS class are distinguishable (Fig. 4.1). Cluster I comprises the biochemically characterized MctC and ActP, for which no Na^+ dependence for solute translocation was observed, whereas cluster II comprises the Na^+ dependent PutP. Members of cluster III have not been characterized biochemically yet. MctP is also H^+ dependent, but did not cluster in any of these subgroups, which correlates with its outstanding substrate specificity. Thus this clustering might reflect ion dependence of the different SSS-type transporters, although further proteins need to be analyzed biochemically to verify this hypothesis.

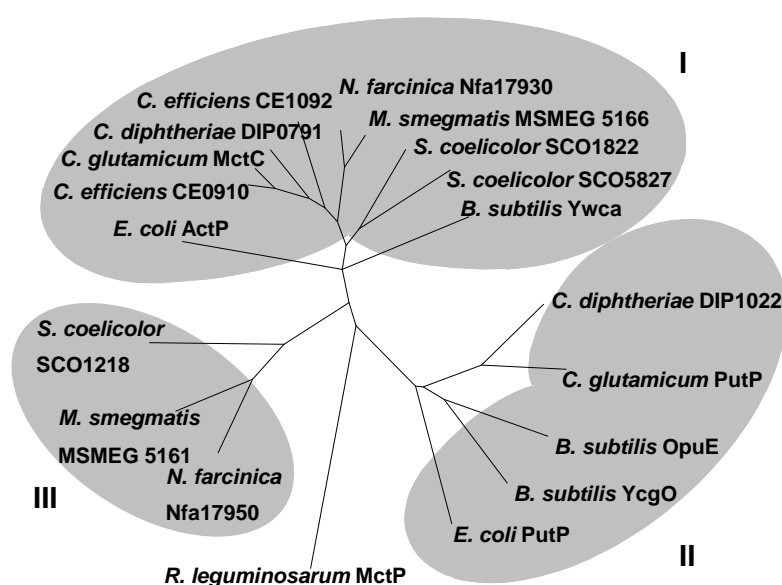


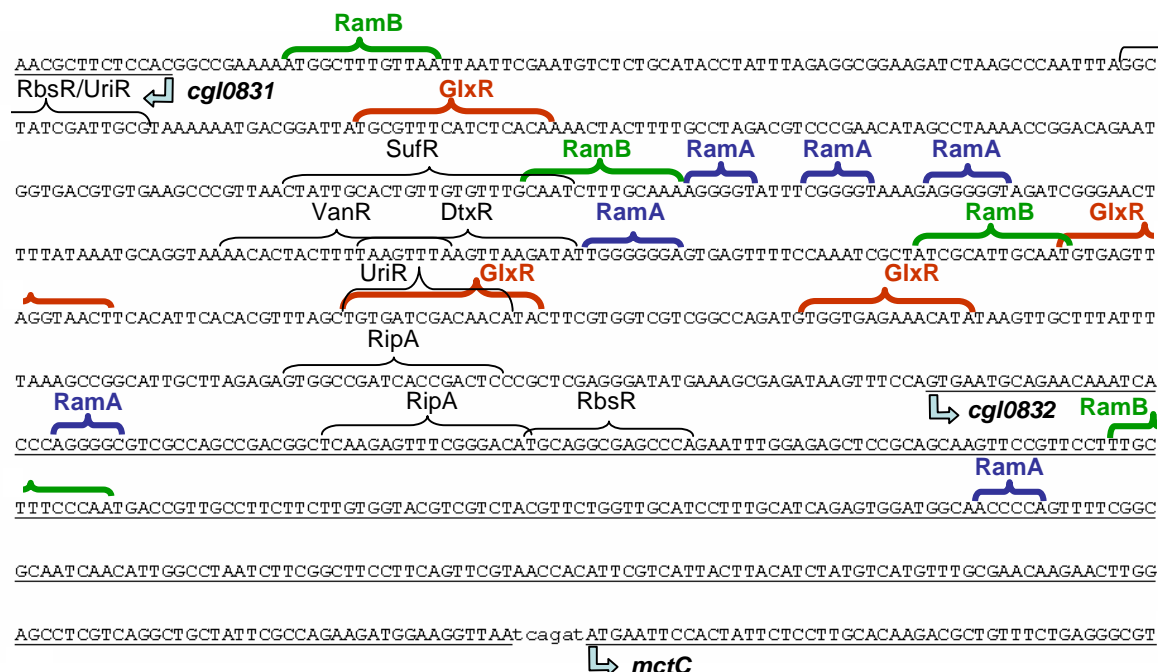
Fig. 4.1 Phylogenetic tree of bacterial putative SSS-type transport systems similar to MctC of *C. glutamicum*. Clusters are indicated by grey shadings.

Another interesting feature of members belonging to cluster I and III is that the corresponding gene directly neighbors a small gene encoding a membrane protein with two transmembrane helices. Co-transcription of this gene in an operon with the one encoding the predicted permease was shown for *yjch-actP* from *E. coli* (Gimenez *et al.*, 2003) and *cgl0832-mctC*. This genetic organization was also observed for the methionine uptake system MetP from *C. glutamicum*, which is co-transcribed with the adjacent gene *cgl1029* encoding a protein with one transmembrane helix. The presence of *cgl1029* was shown to be essential for MetP function (Trötschel *et al.*, submitted), but its function has not been

assigned yet. Its role in substrate translocation, substrate specificity, as a membrane chaperone, or as a regulator is conceivable.

4.1.2 *mctC* expression is regulated by RamA, RamB and a yet unidentified regulator

Transcriptional profiling and activity assays supported the idea of differential expression of both *cgl0832* and *mctC* in dependence on the applied carbon source. Thus, MctC uptake activities were increased upon growth of *C. glutamicum* on carboxylates as compared with activity levels after growth on glucose (table 3.3). The involvement of the global regulators of acetate metabolism RamA and RamB in substrate dependent induction of *cgl0832-mctC* was shown in the respective deletion strains (table 3.4) and by EMSA assays with the promoter region of *cgl0832-mctC* (Jolkver *et al.*, submitted). RamA and RamB regulate genes involved in acetate metabolism, such as *ack-pta* encoding the acetate and propionate activating enzymes acetate kinase and phosphotransacetylase. Moreover, they were shown to regulate genes encoding the glyoxylate cycle enzymes isocitrate lyase and malate synthase (Gerstmeier *et al.*, 2004; Cramer *et al.*, 2006; Cramer *et al.*, 2007). Their regulation of the acetate importer would fit into the global concert of acetate metabolism regulation. However, a closer inspection of *mctC* transcription revealed a dependence on the membrane permeability of the substrate. Thus, the presence of membrane impermeable pyruvate led to double promoter activity (table 3.4) whereas the presence of membrane permeable acetate did not alter the promoter activity of *cgl0832* (table 3.4), and even half of the transcript level of *mctC* was reported from microarray experiments comparing transcription levels during growth on acetate at pH 7.0 and growth on glucose (Eikmanns, personal communication). Surprisingly, concomitant application of the membrane permeable substrates propionate and acetate led to double promoter activity of *cgl0832* (table 3.4) and transcript levels of *mctC* were four times higher during growth on propionate and acetate as compared to growth on acetate (Hüser *et al.*, 2003). The induction upon cultivation on both diffusible substrates seems to contradict the differentiation between diffusible and non-diffusible substrates. Still, this could have been caused by a secondary effect of propionate metabolism. The presence of propionate leads to the formation of several bacteriostatic intermediates. As such, 2-methylcitrate was found to inhibit isocitrate dehydrogenase in *Salmonella typhimurium* (Horswill *et al.*, 2001) and propionyl-CoA inhibited pyruvate dehydrogenase in *Rhodobacter sphaeroides* (Maruyama and Kitamura, 1985) and in *Aspergillus nidulans* the application of propionate was followed by pyruvate excretion (Brock and Buckel, 2004). Although this was not tested in *C. glutamicum*, and pyruvate accumulation was not considered in metabolome analyses during growth on propionate (Plassmeier *et al.*, 2007), a similar effect cannot be excluded. Thus, if propionyl-CoA indeed inhibits the pyruvate dehydrogenase in *C. glutamicum*, pyruvate could have accumulated,



Regarding the promoter region of *cgl0832-mctC* (Fig. 4.2), putative binding sites for several regulatory elements with an E-value of $<10^{-1}$ are identified with the CoryneRegNet database (Baumbach *et al.*, 2006). Several of those were reported to be involved in the utilization of carbon sources or in the regulation of iron-sulfur cluster synthesis, which is in turn important for several carboxylate converting enzymes. In particular, binding sites are predicted for the global cAMP binding regulator GlxR, which was reported to be involved in the metabolism of carbohydrates, carboxylates, aromatic compounds, and nitrogen, the biosynthesis of fatty acids, and deoxyribonucleotides, but also respiration, the cellular stress response, and resuscitation (Kohl *et al.*, 2008). Intracellular cAMP levels were found to be strongly decreased in *C. glutamicum* grown on acetate leading thus to derepression of *aceA* and *aceB* (Kim *et al.*, 2004), which are required for acetate utilization and are also regulated by the regulators of acetate metabolism RamA and RamB (Cramer *et al.*, 2006). RbsR represses the ribose/xylose/arabinose/galactoside ABC-type transport system (Mauzy and Hermodson, 1992), RipA represses aconitase and other iron proteins under iron limitation

(Wennerhold *et al.*, 2005), SufR represses the *suf* gene cluster playing a role in the assembly of iron-sulfur clusters (Nakunst *et al.*, 2007), UriR represses uridine utilization (Brinkrolf *et al.*, 2008), and VanR is a putative transcriptional regulator involved in vanillate metabolism (Brinkrolf *et al.*, 2006). Experimentally, downregulated expression of *cgl0833* was observed in $\Delta dtxR$ (Brune *et al.*, 2006) and surprisingly in $\Delta mcbR$ (Rey *et al.*, 2005), although no binding sites for McbR were predicted in the promoter region of *cgl0832-mctC*. DtxR and McbR are involved in iron and sulphur metabolism, respectively. The involvement of any of these regulators in *mctC* regulation is conceivable; in particular GlxR as a global cAMP dependent regulator. Thus, further studies should focus on binding of GlxR to the promoter region of *cgl0832-mctC* by EMSA studies and the identification of further *mctC* regulators by DNA affinity purification assays.

4.1.3 Role of MctC for growth under natural conditions and for biotechnological applications

The identification of MctC as an acetate-propionate-pyruvate importer enabled insights into carboxylate metabolism of *C. glutamicum* and thus insights for biotechnology. Studies on the $\Delta mctC$ strain allowed the quantification of substrate uptake via active transport and via passive diffusion. Thereby, MctC was shown to be indispensable for pyruvate uptake in *C. glutamicum*, since its deletion caused complete growth inability on this substrate, indicating its membrane impermeability. However, though it is also the only acetate and propionate importer, a growth phenotype on acetate was observed only at very low substrate concentrations. Under natural conditions, low concentrations of acetate or propionate were reported, whereof acetate was found to be more abundant (<1 mM) than propionate (<0.1 mM) (Rothfuss and Conrad, 1993). In this concentration range, the presence of the active uptake system was shown to confer a growth advantage over strains that rely solely on diffusion. On the contrary, if exposed to conditions of low or even neutral pH, *C. glutamicum* cannot avoid uptake of acetate. For *E. coli*, the influx of acetate was shown to introduce pH stress (Nettekoven, personal communication). In order to maintain pH homeostasis, *C. glutamicum* requires H⁺ exporting systems, such as the respiratory chain and yet unidentified proton exporters (Follmann, 2008). H⁺ export against the membrane potential depends on energy, thus causing an additional challenge on the metabolism. This has direct consequences for biotechnological applications. Here, the supply of acetate as a carbon source (usually 1%) has to be considered as extremely challenging with respect to pH homeostasis. On the other hand, the introduction of the gene encoding the uptake system could enhance product yield by either accelerated import of the offered membrane impermeable carbon source or the re-import of a previously excreted by-product. Though continuously aerated, microaerobic conditions are frequently occurring in large fermenters due to high cell densities. This causes the excretion of acetate and thus

leads to carbon loss, not only in *C. glutamicum*, but in many bacteria. In particular, undesired pyruvate excretion was shown to lower the valine and lysine product yield (Blombach *et al.*, 2007a; b). A remedy could be the overexpression of *cgl0832-mctC*, whereby more pyruvate could be taken up again, thus limiting carbon loss and increasing production efficiency. Moreover, the deletion of the importer encoding gene could be advantageous when the re-uptake of the produced compound is not desired. Thus, the production of pyruvate could be improved and the identification of both, pyruvate importer and exporter could promote the utilization of *C. glutamicum* for pyruvate production.

4.1.4 The carboxylate importers DccT and DctA cover a large substrate spectrum

Regarding the transport mechanism and the biological function of dicarboxylate transporters, several classifications can be made. Facultative anaerobic bacteria rely on DcuAB and DcuC type transporters for the uptake or exchange of dicarboxylates at anaerobic conditions. These types of transporters have not been found yet in aerobic organisms, which were shown to import dicarboxylates mostly via DASS (divalent anion:Na⁺ symporter), DAACS (dicarboxylate/amino acid:cation symporter) or TRAP (tripartite ATP-independent periplasmic) type carriers. Biochemically well characterized representatives of each of these classes are SdcS from *Staphylococcus aureus*, DctA from *E. coli* and DctPQM from *Rhodobacter capsulatus* (Hall and Pajor, 2005; Kay and Kornberg, 1971; Forward *et al.*, 1997). Homologues of SdcS and DctA are found in different bacterial classes and also in eukaryots. According to the genome annotation of *C. glutamicum* (Kalinowski *et al.*, 2003; Ikeda and Nakagawa, 2003) the presence of DAACS, DASS, and TRAP-type transporters is predicted, and for fourteen transporters, carboxylates were suggested as their accepted substrate (Winnen, 2005). In the case of succinate, Na⁺ dependent uptake has been reported (Ebbighausen *et al.*, 1991b). Although a homologue of *dctA* and *sdcs* is predicted from its sequence, the laboratory *C. glutamicum* ATCC 13032 strain analyzed in this work did not show growth on dicarboxylates, and thus its uptake abilities came into question, especially considering growth abilities of its widely distributed relative *C. efficiens*. Moreover, this made a systematic approach regarding transporter identification impossible. A deletion of the dicarboxylate importer would not alter the mutant's phenotype on dicarboxylates in comparison to the parental strain.

It was not until Youn's isolation of spontaneous mutants which acquired growth ability that two genes could be assigned as candidates for dicarboxylate uptake. *C. glutamicum* strains isolated upon growth on succinate, fumarate and malate showed higher transcription rates of transporter encoding genes *cgl0225* and *cgl2595* (Youn *et al.*, 2008; Youn, personal communication). Biochemical studies performed in this work proved that DccT (Cgl0225) and DctA (Cgl2595) are indeed transporters with a high affinity towards organic acids. DccT

from *C. glutamicum* shows 42% identity to SdcS and 26% identity to the eukaryotic carboxylate transporter NaDC-1 on amino acid level. Its biochemical features also resemble those of SdcS, which was shown to translocate succinate, fumarate and malate in a Na^+ dependent manner (Hall and Pajor, 2005). The transport was shown to be electroneutral, since 2 Na^+ ions were translocated along with 1 succinate²⁻, which was in contrast to its eukaryotic orthologs, which were shown to couple electrogenic transport of 3 Na^+ to 1 dicarboxylate (Busch *et al.*, 1994; Markovich and Murer, 2004). Moreover, the described carriers showed high affinity for succinate, ranging from 4.5 μM for SdcS to 30 μM for NaDC-3 and 400 μM for NaDC-1 (Pajor, 1994; Steffgen *et al.*, 1999). The affinity of DccT for succinate as determined here was 30 μM , which is roughly in the range of the K_m determined for SdcS. The transport was also shown to be Na^+ dependent, but the varying Hill coefficient for the different C_4 -dicarboxylates ranging from 1.3 for L-malate to 4.6 for fumarate made a clear statement on minimum ion stoichiometry impossible. Furthermore, it was shown that succinate is the preferred substrate of DccT due to slightly lower affinity for fumarate and a 10 times lower affinity for L-malate. Moreover, it mediates the transport of oxaloacetate, as was shown in competition assays and by growth of the *dccT* overexpressing strain on this substrate.

These substrates were also accepted by the second identified *C. glutamicum* dicarboxylate transporter DctA, albeit its affinity ranging between 120 and 730 μM was lower towards all of the determined substrates compared to DccT substrate affinity. Competition assays revealed several further putative substrates of this carrier. These include the dicarboxylates 2-oxoglutarate, oxaloacetate, and L-aspartate, but also citrate, glyoxylate and L-lactate caused a significant reduction of L-malate uptake rates, indicating competition for the binding site. The knock-out of *dctA* caused a severe growth phenotype on L-lactate as sole carbon source and loss of uptake activity for L-lactate. However, DctA mediated lactate transport has not been reported yet, and no indications were given for DctA involvement in lactate metabolism as observed in microarray assays neither during growth on L-lactate nor in the ΔlldR strain, lacking the regulator of lactate metabolism (Stansen *et al.*, 2005; Georgi *et al.*, 2008). An additional problem is the fact that DctA promoted growth of the wild type on dicarboxylates only upon the overexpression of the corresponding gene but growth on lactate does not require additional *dctA* expression. Thus, DctA mediated transport of L-lactate needs to be confirmed by complementation assays in the ΔdctA strain. Moreover, ΔdctA needs to be assessed for LldD activity to exclude effects on L-lactate metabolism besides transport inability.

Comparing the mechanism of DctA and the other dicarboxylate importer DccT of *C. glutamicum*, several further differences in addition to the broader substrate spectrum of DctA were observed. The affinity of DctA towards succinate, fumarate and L-malate is lower than that of DccT and in accordance to the biochemically described DctA system of *E. coli*

(Gutowski and Rosenberg, 1975) the DctA system described here relies on the proton membrane potential and not on Na⁺ ions, as suggested by Ebbighausen *et al.* (1991b). This indicates that Ebbighausen *et al.*, worked on the DccT system, which was not identified at that time, although the determined K_m value of 150 μ M as measured in the presence of 50 mM NaCl corresponds better to the K_m determined here for DctA ($119 \pm 25 \mu$ M) than for DccT ($19 \pm 2 \mu$ M) in the presence of 10 mM NaCl. This could be an indication of the inhibitory effect of Na⁺ on the transporter, when applied in higher concentrations, as was argued for SdcS by Hall and Pajor (2005).

4.1.5 A base transition inhibits growth of the laboratory *C. glutamicum* strain on dicarboxylates

Although *C. glutamicum* was proven to have at least two dicarboxylate importers, the conditions for their expression were not identified, since no growth of the wild type was observed during aerobic incubation on any of the dicarboxylates (Fig. 3.1). However, the spontaneous mutants isolated on succinate, fumarate or malate containing medium all showed a single point mutation in the promoter region of *dccT* or *dctA* (Youn *et al.*, 2008; Youn, personal communication). In the case of *dccT*, a single C \rightarrow T transition at the -15 position and in the case of *dctA* a C \rightarrow T transition at the -12 position in front of the transcription start point possibly caused the altered transcription rates. Indeed, the hereupon resulting sequence AATGT TAATAT TCCCTC -*dccT* and TTTT TATAAT TTTCCG -*dctA* (the altered position is underlined) increases the similarity to the σ^A consensus sequence of the extended -10 region (TGTGC/G TATAAT GG; Patek, 2005). σ^A is one of the 7 sigma factors identified in *C. glutamicum* according to its genome annotation (SigA (σ^A), SigB, SigC, SigD, SigE, SigH and SigM) and it is the principle sigma factor required for the transcription of most housekeeping genes (Halgasova *et al.*, 2001). The SigB recognition sequence is highly similar to that of SigA (Nesvera and Patek, 2008), but was found to drive transcription of genes relevant for the transition of exponential to stationary growth phases as well as of genes in the stationary phase (Larisch *et al.*, 2007) or during environmental stresses such as acid stress, ethanol shock, and cold shock (Halgasova *et al.*, 2001). The other sigma factors recognize distinct motives and mostly regulate the transcription of genes with extracytoplasmic functions or those involved in the acclimatization to various stress conditions (Helmann, 2002; Nesvera and Patek, 2008). Thus, the point mutation might lead to better recognition of the promoter region by the RNA polymerase Eo^A or Eo^B holoenzyme and consequently to more frequent transcription initiation, causing increased presence of the respective transporter and thus growth on dicarboxylates. In addition, the altered promoter sequence might have promoted improved recognition by a transcriptional activator, or on the contrary, declined recognition by a repressor. Indeed, the affected region of *dccT* lies within the proposed binding site of UriR, which was shown to bind to cre-like (catabolite-

responsive element) sequences. Up to now, it has been reported to be stimulated by the presence of either uridine or ribose (Brinkrolf *et al.*, 2008). For *dctA*, an ArgR binding site is predicted for this region. ArgR was reported to regulate the *argCJBDFR* operon, which is involved in arginine metabolism (Makarova *et al.*, 2001). Since neither of the regulators was reported to be involved in carboxylate metabolism, their involvement in *dccT* or *dctA* regulation seems unlikely.

Bacterial regulation of carboxylate metabolism in response to their presence in the medium is mediated by the two-component system families DcuSR, DctBD, DctSR, or CitAB (Janausch *et al.*, 2002). The regulatory mechanism of two-component systems was found in nearly all bacterial species (Nixon *et al.*, 1986) and comprise a membrane-bound dimeric sensor histidine kinase and a cytoplasmic response regulator. According to the genome annotation, 13 putative two-component systems have been identified in *C. glutamicum*: CgtSR1, 2, 4-11, CitAB, MtrBA, and PhoSR. MtrBA was shown to be involved in osmotic and chill stress response (Möker *et al.*, 2004; Möker, 2006) and PhoSR is required for acclimatization of *C. glutamicum* to phosphate limiting conditions (Kocan *et al.*, 2006). The CitAB system was shown to control the uptake and metabolism of citrate in *Klebsiella pneumoniae* (Bott *et al.*, 1995), and it is required for the induction of the citrate importers CitM and TctABC in *C. glutamicum* (Schaffer *et al.*, unpublished). However, none of the two-component systems that are important for dicarboxylate utilization were found in *C. glutamicum* and no CitB binding sites are predicted neither in the upstream region of *dccT* nor *dctA*. A starting point for the identification of regulator candidates might be the finding that the newly identified dicarboxylate importer DcsT from *C. glutamicum* R, which is to 86% identical to DccT from *C. glutamicum*, was found to be expressed only in the early exponential growth phase, but was repressed in the later growth phases or in the presence of glucose (Teramoto *et al.*, 2008). This suggests the involvement of cAMP dependent regulators, such as e.g. GlxR. Whether this holds true for DccT remains to be seen. *dccT* transcription was induced upon cultivation on lactate (Youn *et al.*, 2008), but the predicted binding site for LldR – the regulator of the lactate utilization operon comprising a permease and the L-lactate dehydrogenase encoding genes *cgl2917-lldD* (Georgi *et al.*, 2008) – was unaffected by the point mutation. An LldR binding site is also predicted in the unmodified promoter region of *dctA*. There were strong indications presented in this work for the idea that DctA acts not only as a dicarboxylate but also as an L-lactate importer. Its regulation by LldR would support its participation in L-lactate metabolism. On the other hand, the involvement of LldR in the regulation of these two dicarboxylate importers would extend its function from the regulator of L-lactate utilization operon to a putatively global regulator of carboxylate metabolism in *C. glutamicum*. However, expression profiles of a Δ *lldR* strain and a strain overexpressing *lldR* indicated differential expression of *cgl2917-lldD* only (Georgi *et al.*, 2008), challenging the idea of LldR involvement in *dctA* or *dccT* regulation. So, further

studies regarding the conditions that promote *dccT/dctA* expression and the regulators involved in this process (in particular LldR) are required to understand dicarboxylate metabolism of *C. glutamicum* and its inability to grow on dicarboxylates, although it is equipped with at least two different dicarboxylate uptake systems. In particular, DNA affinity purification and EMSA studies should be considered. The investigation of dicarboxylate uptake regulation in *C. efficiens* can also be considered, since it showed growth on all dicarboxylates applied but lacks a DctA-like protein. Whether dicarboxylate uptake occurs via a DccT homologue YS314, which showed 77% sequence identity remains to be seen. Mapping the transcriptional start point and determining the sigma factor binding region as well as the identification of regulators in this organism could shed further light on dicarboxylate metabolism regulation. Furthermore, an interesting aspect would be the comparison between the laboratory *C. glutamicum* ATCC 13032 strain and a newly isolated strain from soil, in particular regarding their growth ability on dicarboxylates and the promoter region of *dccT* and *dctA*. Decades of laboratory cultivation usually on glucose might have caused an accidental isolation of a strain which had the previously mentioned T à C transition, but the original wild type might have had the same promoter sequence as the spontaneous mutants which were able to grow on dicarboxylates. This would shed further light on the regulation of dicarboxylate metabolism in *C. glutamicum*.

The understanding of dicarboxylate metabolism of *C. glutamicum* would be of particular interest for industrial applications. The ability to modulate dicarboxylate uptake and thus the re-uptake of excreted dicarboxylates as succinate during conditions of low oxygen supply would reduce the need for anaplerotic reactions (Menkel *et al.*, 1988) during e.g. lysine production and could thus increase the product yield.

4.2 Carboxylate export

Efflux of metabolites has been observed in many bacteria and it is a prerequisite for biotechnological production. In spite of that, the corresponding exporter systems have been identified only for a limited number of substrates. Regarding carboxylate export, the lactate:malate or lactate:citrate antiporters MleP, MaeN, and CitP have been identified (Bandell *et al.*, 1998; Wei *et al.*, 2000; Marty-Teyssset *et al.*, 1995). Moreover, the export of acetate via the AatA comprising ABC system has been suggested (Nakano *et al.*, 2006). Regarding *C. glutamicum* in particular, only the amino acid exporters LysE, ThrE, and BrnFE have been identified (Vrljic *et al.*, 1996; Simic *et al.*, 2001; Kennerknecht *et al.*, 2002). No carboxylate exporters were known at the beginning of this work, although the export of lactate, succinate, acetate, malate, and pyruvate was observed (Inui *et al.*, 2004, Menkel *et al.*, 1988; Blombach *et al.*, 2007a). Also the mechanisms of substrate export are not clear. However, thermodynamic considerations strongly suggest the existence of active transport systems.

C. glutamicum maintains a relatively constant internal pH of 7.0-8.5 even at changing external pH values of 6.0-9.5 (Follmann, 2008). The exposure to oxygen deprived conditions leads to bulk lactate export but internal pH values remain constant at pH 7.5 (Follmann, personal communication). The pKa of all carboxylic acids discussed here is below this value. Consequently, the acid is present in its dissociated form and only minor amounts are protonated and thus potentially membrane permeable. At a cytosolic pH of 7.5, 0.0008% of pyruvic acid and 0.0224% of lactic acid are in their protonated state.

The efflux of a substrate can be mediated by different mechanisms which require different amounts of energy. This can be diffusion, primary or secondary active transport. Depending on the biosynthetic pathway, the export of the negatively charged carboxylate can require the export of at least one proton to avoid acidification of the cytoplasm. This does not necessarily need to occur via the same transport system, but is an additional transport event requiring additional energy, since it has to overcome the inwardly directed membrane potential. Thus, the overall driving force for carboxylate export can consist of the concentration gradient, $\Delta\Psi$, ΔpH or ATP hydrolysis if a primary active transporter is involved either for carboxylate or proton export (Fig. 4.3).

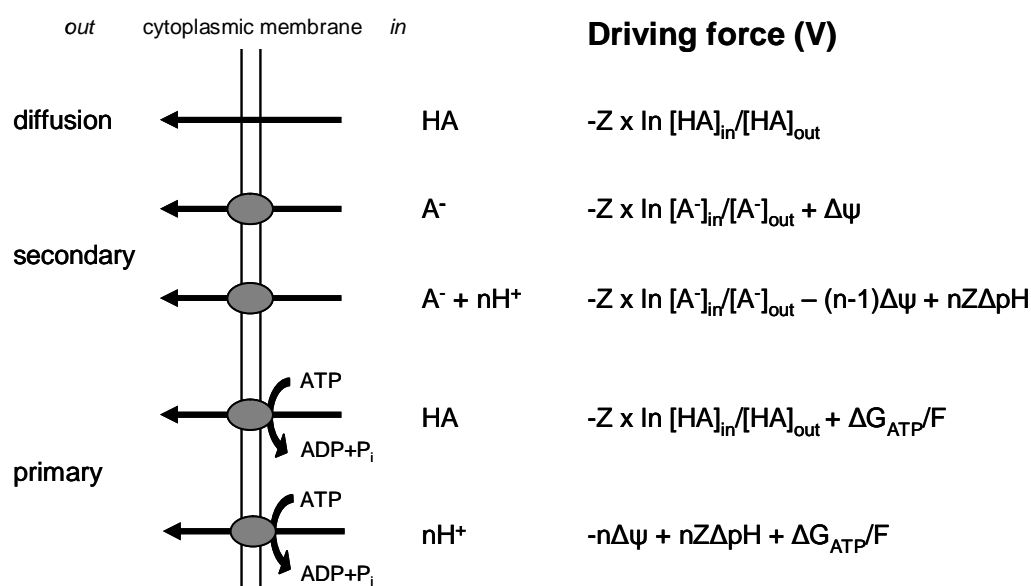


Fig. 4.3 Schematic overview of mechanisms involved in organic acid export and the required driving force in volts (V). $Z = (R \times T)/F$ and R = gas constant; T = absolute temperature; F = Faraday constant. (Adapted from van Maris *et al.*, 2004b).

According to these driving forces, the ratio of intracellular over extracellular total acid (HA_{tot}) (undissociated + dissociated) that is required to enable a particular mode of export can be calculated. For *C. glutamicum*, extensive research has been performed on the determination of $\Delta\Psi$ and ΔpH at different external pH values (Follmann, 2008) and the data was used here for the determination of the $[HA_{tot}]_{in}/[HA_{tot}]_{out}$ ratio, which is required for a certain transport mechanism. The calculated ratio is depicted in Fig. 4.4.

Electroneutral efflux via a transporter or via diffusion can be driven only by the concentration gradient of the substrate. The threshold value for $[HA_{tot}]_{in}/[HA_{tot}]_{out}$ above which net export of the undissociated acid might be mediated by diffusion can be calculated according to the equation for the driving force indicated in Fig. 4.3. Obviously, the only decisive element in the equation is the concentration gradient of the protonated acid, which is dependent on its pKa and thus the pH value of the medium. However, an internal pH value of approx. 7.5 causes an almost complete dissociation of the acid and only a small fraction is protonated and thus decisive for acid efflux. Consequently, the amount of the total acid (dissociated + undissociated) has to be increased, in order to maintain an outwardly directed concentration gradient of the protonated form. On the other hand, the external pH also influences the dissociation grade of the acid and thus the required internal concentration of the protonated form to maintain an outwardly directed concentration gradient. Considering these parameters, a ratio of the total acid $[HA_{tot}]_{in}/[HA_{tot}]_{out}$ which is required for diffusion mediated efflux can be calculated and is indicated by the green line in Fig. 4.4. The presented ratio is determined for lactate with a pKa of 3.86, but comparable values were obtained for carboxylates of a slightly different pKa, for example pyruvate with a pKa of 2.39 (not shown).

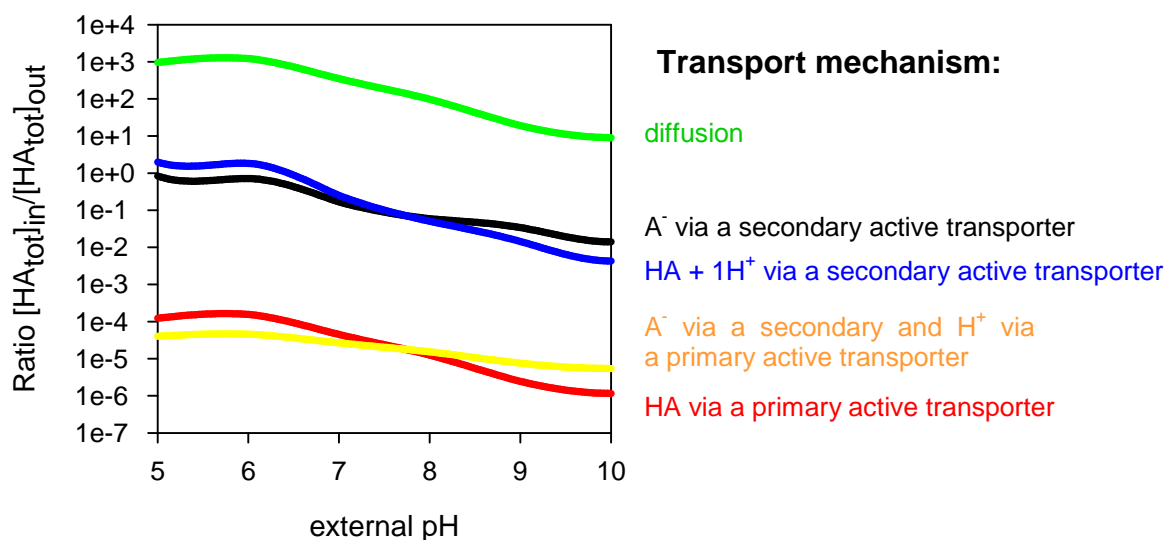


Fig. 4.4 The ratio of intra- and extracellular total acid ($HA+A^-$) required for the efflux of the substrate by different mechanisms. Calculations were performed for lactic acid ($pK_a=3.86$) and the actually measured $\Delta\Psi$ and ΔpH values in *C. glutamicum* at different external pH (Follmann, 2008). Free energy of ATP hydrolysis was assumed to be -40 kJ and constant over the pH range. The lines represent the value for $[HA_{tot}]_{in}/[HA_{tot}]_{out}$ ratio that is required for thermodynamic equilibrium. All export mechanisms indicated by the lines below a certain $[HA_{tot}]_{in}/[HA_{tot}]_{out}$ ratio are possible. (following van Maris *et al.*, 2004b)

If the ratio is below the indicated threshold, the net export of the substrate requires additional energy either in form of the electrogenic components $\Delta\Psi$ and ΔpH or, if the ratio becomes even lower, substrate export can occur only via an ATP dependent transporter.

These thermodynamic considerations illustrate the importance of an active export system. Its presence can be predicted by the quantification of internal and external substrate concentrations and even more straightforward by the characterization of the membrane permeability for a certain substrate. Import studies showed that the *C. glutamicum* membrane is impermeable for lactate and pyruvate and since the conditions for their export were successfully set here, a part of this work was dedicated to the identification of the pyruvate and lactate export systems.

4.2.1 The redox state could be decisive for the excretion of the fermentative end-products lactate and succinate

Being deprived of the terminal electron acceptor oxygen or nitrate, *C. glutamicum* converts glucose almost completely to lactate and in minor amounts to acetate and succinate, which are subsequently excreted (Fig. 3.18). Thus, 2 NAD⁺ per 2 NADH produced in glycolysis are restored by the reduction of pyruvate to lactate. The maintenance of glycolysis under oxygen deprived conditions is decisive for ATP formation, yielding a net of 2 ATP per glucose molecule. Furthermore, the conversion of pyruvate to acetyl-CoA and acetate also leads to the production of 1 ATP, but under dispense of one additional NAD⁺. This might explain why acetate is only a minor fermentative end-product of this bacterium. Furthermore, *C. glutamicum* was shown to follow the reductive path of the TCA cycle, mostly beginning with the carboxylation of PEP to oxaloacetate and its subsequent reduction to malate, which is then decarboxylated to pyruvate via the NADP⁺ dependent malic enzyme (Dominguez *et al.*, 1998). Thereby, one additional NAD⁺ and NADPH are gained for anabolic reactions such as glutamate synthesis. Moreover, malate can be further converted to fumarate and succinate, which is subsequently excreted (Inui *et al.*, 2004). So far, no fumarate respiration was observed in *C. glutamicum* and of the *E. coli* operon *frdABCD* encoding the fumarate reductase, only proteins showing a significant identity to FrdA, the flavoprotein subunit, and FrdB, the iron-sulfur protein subunit, can be found. On the other hand, fumarate reductase can be replaced by anaerobically expressed succinate dehydrogenase in *E. coli* (Maklashina *et al.*, 1998). Thus, the reverse mode of action of succinate dehydrogenase in *C. glutamicum* can be assumed.

The export of the above mentioned fermentative end-products proceeds most likely via active export systems, which were sought in this work. The requirement of DctA or DccT for growth on succinate demonstrates the need of an active transporter for sufficient substrate uptake and an export system is likely to be present for its efflux. Even for the membrane permeable substrate acetate, the presence of an exporter in *Acetobacter aceti* has been demonstrated (Matsushita *et al.*, 2005; Nakano *et al.*, 2006). Regarding the high external lactate concentrations of up to 140 mM, approx. 10 times higher internal lactate concentrations would be required at an external pH of 6.0 (Fig. 4.4), if lactate efflux is

mediated solely by diffusion. This has not been observed yet. Thus, either a primary or a secondary active lactate export system must be present in *C. glutamicum*. Secondary active lactate exporters were observed in *S. cremoris* (Otto *et al.*, 1980), but the corresponding protein has not been identified yet. An antiport mechanism of lactate with malate or citrate, as has been observed for *L. mesenteroides* or *L. lactis* (Marty-Teyssset *et al.*, 1995; Bandell *et al.*, 1998) is unlikely to drive lactate export, since *C. glutamicum* excretes lactate into the medium without the need of the addition of further substrates.

In the course of the work, several approaches were made in order to identify the lactate exporter. In the first place, the genetic organization and regulation of the lactate dehydrogenase encoding *ldhA* was considered. In contrast to several other bacteria, no transporter encoding gene was found to be transcribed in an operon with *ldhA* in *C. glutamicum*. The regulation of *ldhA* has been recently proposed to be mediated by the cAMP dependent global regulator GlxR (Kohl *et al.*, 2008). The regulation of lactate export by GlxR can thus be assumed. Binding sites of GlxR can be found in the promoter regions of several genes encoding putative transporters: *cgl0769*, *cgl1005*, *cgl1566*, *cgl1568*, *cgl2908*, *cgl2912*, *cgl2917*, *pcaK*, *narK*, *vanK*, *genK*, *gluCD*, *ptsF*, and *ptsG*. Of these, VanK has been shown to import vanillate, GenK to import gentisate, PcaK to import 4-hydroxybenzoate and protocatechuate, and BenE and BenK to import benzoate (Chaudhry *et al.*, 2007). GluCD is part of a glutamate importer (Kronmeyer *et al.*, 1995) and the PTS transporters catalyze sugar uptake (Moon *et al.*, 2007). Furthermore, there are strong indications that NarK catalyzes nitrate/nitrite transport (Nishimura *et al.*, 2007) and Cgl2917 was proposed to import lactate (Stansen *et al.*, 2005), although this idea is challenged by the data on lactate import presented here (conf. 3.3). Of the remaining putative transporters, Cgl1005, Cgl1568, Cgl2908, Cgl2912, and Cgl2917 have been tested for lactate export but showed no differences to the wild type regarding lactate accumulation rates (data not shown). Mutants of *cgl0769* and *cgl1566*, both encoding hypothetical membrane proteins with 4 and 14 transmembrane helices, respectively, have not been created yet, and are still good candidates for putative lactate exporters.

Since this approach failed to indicate a putative lactate exporter, the search was expanded on genes putatively transcribed in an operon with *ldhA* in other bacteria. Thereby, several putative transporter encoding ones were found that showed significant homology to *C. glutamicum* transporter encoding genes. Indeed, the knock-out of one of these candidates caused a severe phenotype of *C. glutamicum* being exposed to oxygen deprivation conditions (Fig. 3.22). The accumulation of succinate observed in the *icgl2211* strain argues for the involvement of Cgl2211 in succinate export. If succinate accumulation reduces the turnover capacity of the reductive TCA cycle, a perturbation of the cellular redox status is a conceivable consequence. The maintenance of the proper NAD⁺/NADH ratio is of extreme importance for sustaining the glucose consumption. Slight imbalances of this ratio

were demonstrated to have negative effects on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity in *C. glutamicum* (Dominguez *et al.*, 1998), leading to an arrest of glycolysis. The consequent lack of pyruvate leads to decreased lactate and acetate formation, which was observed in the *icg/2211* strain. In the wild type, the NAD^+/NADH ratio might additionally regulate succinate efflux via the NAD(H) binding TrkA domain of Cgl2211. This regulation mechanism has already been shown for K^+ uptake by the Trk system, where pore opening of the KTN domain is caused by TrkA in response to NADH binding (Schlösser *et al.*, 1993; Roosild *et al.*, 2002). In the case of Cgl2211, a low NAD^+/NADH ratio might indicate the need for additional NADH oxidation and thus promote succinate efflux in order to maintain the flux via the reductive TCA cycle. However, further experiments are required to validate Cgl2211 as the succinate export system as well as to characterize the export biochemically and to determine the regulatory mode of action of the TrkA domain. Next to the NAD(H) binding TrkA domain, Cgl2211 possesses 2 AAE domains (Aspartate:Alanine exchanger), which are conserved in orthologues of AspT (Nanatani *et al.*, 2007). AspT of *Lactobacillus* subsp. M3 and *Tetragenococcus halophila* is assumed either to import the dicarboxylate aspartate in a H^+ dependent manner, or to catalyze the electrogenic exchange of aspartate and alanine (Abe *et al.*, 1996; 2002). Thus, a direct analogy of a dicarboxylate export is not given, putting the function of Cgl2211 in *C. glutamicum* as a succinate exporter into question. Another interesting aspect is the genetic organization of *ldhA* and the *cgl2211* homologue H16_A1680 in *R. eutropha*, which are presumably transcribed in an operon. Whether *ldhA* and *cgl2211* are also transcribed concomitantly in *C. glutamicum* by the action of the same regulator would promote the understanding of carboxylate metabolism and the regulatory network involved. A closer inspection of the *cgl2211* promoter region revealed a putative GlxR binding site. Since GlxR was previously shown to regulate *ldhA* transcription (Kohl *et al.*, 2008), it is most likely involved in the regulation of genes which are important for metabolism under oxygen deprived conditions.

Next to the previously discussed reverse genetics approach, a screen of the large mutant collection was carried out in order to identify a putative lactate exporter. This screening assay revealed the importance of phosphoglucosomerase for lactate production during oxygen deprivation conditions (Fig. 3.29), which has also been described for the lysine producing *C. glutamicum* DSM5715 strain (Marx *et al.*, 2003). The Δpgi strain is restricted to the pentose phosphate pathway for glucose turnover. During aerobic conditions, the increased formation of NADPH is beneficial for anabolic reactions such as lysine biosynthesis (Marx *et al.*, 2003) but when the oxidation ability is restricted due to the lack of oxygen, increased NADPH levels additionally affecting the redox state might consequently inhibit GAPDH, and thus disrupt the glycolytic path towards lactate. On the other hand, the deletion of *pgi* caused rapid degradation of *ptsG* mRNA due to the action of the small RNA SgrS, thus reducing glucose uptake in *E. coli* (Kimata *et al.*, 2001; Wadler and Vanderpool,

2007). A similar mechanism can thus be assumed for *C. glutamicum*, which would have also caused reduced lactate production.

Further insights into carboxylate metabolism at microaerobic conditions could have been gained by the identification of the lactate exporter. This task was not accomplished here, since no lactate exporter candidate was identified neither by rational candidate selection nor by a broad screening assay. However, two candidate genes still remain to be tested due to the predicted regulation by GlxR. Additionally, the integration site has not been determined yet for two mutants carrying the transposon. Besides mutants affected in the lactate exporter, the detection of mutants being deficient in lactate synthesis was expected. Those were strains affected in the gene encoding the lactate dehydrogenase or the phosphoglucosomerase (PGI). The detection of two mutants being affected in *pgi* demonstrates that the screening assay worked in principle and could be utilized for the detection of mutants with a lactate export or synthesis phenotype. However, the screening enabled only the differentiation of severely reduced lactate production over the whole time scale of the test. Initially reduced export rates due to the lack of the sought carrier would not have been detectable, if another system takes over the lactate export function. Thus, if *C. glutamicum* has several exporters that translocate lactate, none of them will be detected in the designed test.

The failure to detect a lactate exporter candidate suggests the presence of more than one lactate exporter. To test this, a thorough investigation of lactate export mechanism is required. The reduction of the assay duration would enable the application of transport uncoupling agents. The determination of the transport mechanism as well as the number of export systems involved would become possible then. One solution might be to use an even denser culture than that of an OD₆₀₀ of 20-30, causing quicker oxygen consumption and thus quicker accumulation of lactate in detectable concentrations. An even more sensitive detection method is the detection of ¹⁴C-labelled substrates. Under microaerobic conditions, most of the glucose is converted to lactate. Offering ¹⁴C-labelled glucose, the accumulation of ¹⁴C within the cells upon the application of a carrier inhibitor might indicate impaired transport and thus enable conclusions on its mechanism. Another problem to circumvent in shorter assays is the need for the quick setting of oxygen deprivation conditions. Additional application of oxygen scavengers might be tested, if they do not interfere with cellular metabolism in time scales of the assay. Furthermore, if the expression of the lactate exporter(s) is induced upon oxygen deprivation, microarray experiments can be performed in order to indicate a transporter candidate. This has already been accomplished for *C. glutamicum* R (Inui *et al.*, 2007) and two putative transporter encoding genes, which were induced in this strain, were also found in *C. glutamicum* ATCC 13032. Those are the ABC-type transporter with the ATPase and permease subunit Cgl1147 and an MFS type transporter Cgl2978, which still need to be tested for their involvement in lactate export.

4.2.2 Pyruvate overflow enhances its utilization and export

Besides the well-known export of fermentative end-products, the excretion of the central metabolite pyruvate can be triggered in *C. glutamicum* upon the deletion or inhibition of pyruvate dehydrogenase (PDH). So, the compound 1-aminoethylphosphinate (AEP) was found to inhibit PDH upon its deamination in *Klebsiella pneumoniae* (Laber and Amrhein, 1987). Moreover, propionyl-CoA was shown to inhibit PDH by competing with CoASH in *Rhodobacter sphaeroides* (Maruyama and Kitamura, 1985) and was discussed to act also on *C. glutamicum* (conf. 4.1.2). Moreover, pyruvate export was also observed during cultivation of *C. glutamicum* on lactate (Cocaign *et al.*, 1993).

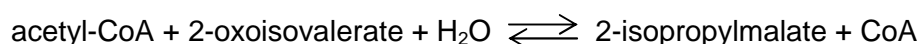
A pyruvate exporter is needed due to the membrane impermeability of this substrate. This has been observed in uptake measurements, and is also indicated by the complete loss of growth ability upon the deletion of the pyruvate importer MctC (conf. 3.2.1). Thermodynamic considerations also suggest the presence of an active exporter. As described for the Δace strain lacking the pyruvate dehydrogenase, approx. equal intra- and extracellular pyruvate concentrations were observed (Blombach *et al.*, 2007a), requiring the presence of an active export system to maintain efflux at the particular pH set in the experiment.

The quest for the pyruvate exporter is complicated by the fact that (I) no bacterial exporter is known to accept pyruvate and (II) the regulation of pyruvate metabolism has barely been studied. Thus, no pyruvate exporter could be suggested from homology analyses or the inspection of putative regulator binding sites. The only approach to find a pyruvate exporter candidate was either a screen, being only successful, if only one export system is present in *C. glutamicum*, or the analysis of differential gene expression upon the setting of pyruvate exporting conditions, which was conducted here and is described in 3.5.4. The application of AEP was found to influence the expression rates of several genes (table 7.4). As shown by the microarray analysis, the largest fraction of downregulated genes was involved in translation, encoding subunits of the ribosomal complex, indicating an arrest of overall translation upon application of AEP. Among the upregulated genes were several directly involved in pyruvate metabolism utilizing it, such as *aceE*, encoding the 2-oxoacid decarboxylase subunit of the pyruvate dehydrogenase; *pqo*, encoding pyruvate:quinone oxidoreductase, and *ldhA*, encoding lactate dehydrogenase. This reflects the mechanisms that the cell applies to cope with increasing pyruvate concentrations. Moreover, the fructose-bisphosphate aldolase encoding gene *fda* was also slightly induced, suggesting increased flux via the pentose phosphate pathway. The fatty acid metabolism was also altered, as indicated by increased transcription of genes encoding acetyl-CoA carboxylase, a long chain-fatty acid-CoA ligase, and a CoA-transferase.

Regarding the transcription of transporter encoding genes, only 8 genes encoding transporters were found to be induced including a transporter of unknown function, and *putP*

encoding a proline importer. Unfortunately, none of them was found to serve for pyruvate export under the tested conditions.

Differentially expressed transcriptional regulators might provide a clue about the metabolism and putative pyruvate exporters under pyruvate accumulating conditions. Among the detected putative transcriptional regulators, only RamA has been thoroughly investigated and functionally assigned as the regulator of acetate metabolism, being required for the induction of genes encoding enzymes for acetate utilization (Cramer *et al.*, 2006). Regarding the fact, that RamA also regulates *mctC*, encoding the pyruvate importer, its downregulation might consequently reduce *mctC* expression, avoiding additional pyruvate uptake. However, this idea cannot be confirmed, since *mctC* transcript was not detected in the microarray (table 7.4). Another upregulated regulator was RipA, which has been shown to regulate a number of genes, including those encoding aconitase, succinate dehydrogenase, the nitrate/nitrite transporter and nitrate reductase, isopropylmalate isomerase, catechol 1,2-dioxygenase, phosphotransacetylase, and catalase under iron limitation conditions (Wennerhold *et al.*, 2005). The induced transcription of the corresponding gene correlates with the repression of *leuD*, which encodes isopropylmalate isomerase, which is required for L-leucine synthesis. On the contrary, the highest induced gene under pyruvate production conditions was *leuA*, encoding 2-isopropylmalate synthase, which is also decisive for L-leucine synthesis. This enzyme catalyzes the reaction:



2-oxoisovalerate is derived through a couple of reaction steps from pyruvate and is either converted to L-valine by valine:pyruvate aminotransferase or subsequently converted to L-leucine starting from 2-isopropylmalate. The reduced occurrence of LeuD required for L-leucine synthesis points at a directed flux towards L-valine. Indeed, only an increased L-valine and L-alanine production was observed in the Δace strain (Blombach *et al.*, 2007a). However, the induction of *leuA* during pyruvate overflow indicates also a possible flux towards L-leucine, although its synthesis has not been reported under pyruvate producing conditions yet. Thus, this enzymatic equipment represents another means to regulate internal pyruvate concentrations, increasing the flux towards the synthesis of amino acids derived from pyruvate.

The other regulators which were found to be induced have not been characterized in *C. glutamicum* yet. Two putative regulators are annotated to belong to the ArsR family, of which two members are thought to play a role in arsenic resistance of *C. glutamicum*, which is conferred by two arsenite permeases and putative arsenate reductases (Mateos *et al.*, 2006). Their regulation by ArsR1 and ArsR2, respectively, was suggested due to the close genetic localization of the corresponding genes. The genetic organization of the two genes encoding the two putative transcriptional regulators of the same family that were induced upon AEP treatment, is distinct from the *arsR1/arsR2* organization, and no membrane

protein encoding genes were found in their vicinity. Thus, no conclusions on putative transporter encoding genes being regulated by these could be drawn. MerR-type regulators also promote heavy metal resistance (Hobman, 2007) but have not been characterized in *Corynebacteria* yet. However, heavy metal resistance is frequently conferred by the action of efflux pumps of the RND family, which accept a broad range of metals, drugs and organic components such as antibiotics, siderophores or quorum sensing signals (Nies, 2003). Indeed, 2 of the induced transporter encoding genes were annotated as ABC-type multidrug efflux system TetB and an ABC-type siderophore transport system, respectively. The multidrug exporter TetB accepts structurally different antibiotics such as tetracycline, oxytetracycline and oxacillin (Tauch *et al.*, 1999). However, the role of these transporters under the tested conditions is not clear, since the knock-out of these transporters did not cause an alteration in pyruvate export. Whether any of them was catalyzing the export of AEP or of the deamination product acetylphosphinate or even the export of any of the other metabolites that are likely to be synthesized as L-lysine or lactate, remains to be seen.

In summary, the microarray performed under pyruvate producing conditions revealed the acclimatization of *C. glutamicum* to pyruvate overflow by directing metabolic fluxes to substrates derived from pyruvate. Also several transporter encoding genes showed altered expression, but their knock-out did not cause altered pyruvate export under the tested conditions.

However, the experiment gave signals for only a quarter of all corynebacterial genes, and no conclusions can be drawn on the expression levels of the missing genes. As already discussed in 4.1.2, a further PDH inhibitor might be propionyl-CoA, which is formed upon the cultivation on propionate. A transcriptional profile comparing gene expression during growth on acetate and acetate with propionate is available (Hüser *et al.*, 2003). However, among the 117 genes induced during growth on acetate and propionate, only 13 were also induced upon the application of AEP. These genes encode enzymes involved in fatty acid metabolism (DtsR1 and FadD2), the AceE subunit of the PDH, 7 genes of unknown function but whose gene products are predicted to have two or less transmembrane helices, and 3 putative transporter proteins. Those were the proline transporter PutP, Cgl0679, and Cgl2942. Neither of them was found to be involved in pyruvate export. On top of that, the putative transporter encoding genes *cgl1281* and *cgl0101* were 5.66 and 1.71 times induced, respectively, during incubation on propionate as additional carbon source. The knock-out of *cgl1281* did not cause an alteration of pyruvate export upon application of AEP (data not shown), but the *icgl0101* strain still needs to be tested. However, the low overlap of induced genes upon application of AEP and during cultivation on acetate and propionate indicates that these two conditions have different impacts on metabolism and the inhibitory action of propionate application on PDH still needs to be proven.

Instead of working in that direction, the search of a putative pyruvate exporter was continued by analysing genes which might be transcribed in an operon with those found to be induced in the microarray performed upon AEP application. Indeed, first results with strains being defective in Cgl0231 and Cgl0324, as well as the knock-out of *cgl0267* (*cpaA*) and *cgl2917*, which is co-transcribed with *lldD*, the L-lactate utilizing enzyme, showed a change in pyruvate efflux.

According to the genome annotation of *C. glutamicum*, Cgl0231 is predicted to be a drug exporter of the RND superfamily, which exports a variety of different substrates in antiport with H⁺. Moreover, the *cgl0231* promoter region shows binding sites for RamB and for no other regulators involved in carboxylate metabolism, which supports the idea of its role in pyruvate metabolism. However, the phenotype of *icgl0231* upon addition of AEP rather suggests that Cgl0231 is required for AEP export, which influenced this strain's metabolism, leading to reduced glucose consumption. Unfortunately, no experimental data is published for this protein or a homologue of it in other bacteria.

Cgl0324 is annotated as a membrane protein, and no further details can be given from homology searches. For *cgl0324*, binding sites for RamB, RipA, LldR, ArsR1, ArsR2, and AcnR, which also regulates the aconitase encoding gene (Krug *et al.*, 2005) are predicted. Thus, this candidate might be the sought pyruvate exporter.

CpaA is annotated as a multicomponent K⁺:H⁺ antiporter and its homologues were shown to be important for acclimatization to alkali stress (Putnoky *et al.*, 1998), and also for choline, cholate and Na⁺ efflux (Ito *et al.*, 1999; 2000), but the export of negatively charged substrates (such as pyruvate) has not been reported. For *C. glutamicum*, no indications for its involvement in acclimatization to pH stress were found, but its deletion caused reduced resistance against high salt (NaCl, KCl) concentrations (Follmann, 2008; Yurtsever, 2008). Moreover, its promoter region contains binding sites only for RipA and DtxR, and for no further regulators of carboxylate metabolism. Thus, the involvement of CpaA in pyruvate metabolism seems unlikely, but needs to be investigated further on.

The best analyzed pyruvate exporter candidate is Cgl2917, which was proposed to be a lactate permease. It is transcribed in an operon with *lldD* encoding the L-lactate utilizing lactate dehydrogenase (Stansen *et al.*, 2005) and its regulation by the regulator of lactate metabolism LldR was directly shown (Georgi *et al.*, 2008). Considering the fact, that cultivation on L-lactate caused pyruvate overflow (Cocaign *et al.*, 1993), the concomitant expression of genes encoding the L-lactate utilizing LldD and the pyruvate exporter is conceivable, if Cgl2917 played a role as a valve under these conditions.

The phenotype of these mutants as well as bioinformatic predictions and experimental data indicate the involvement of these proteins in pyruvate export. However, further experiments are required to validate this hypothesis. In particular, the quantification of internal substrates is required to support the idea of any of the mentioned candidates as a pyruvate exporter.

Further work should be addressed on the mechanism of pyruvate export. The *in vivo* characterization of pyruvate efflux is complicated by the relatively extended time scale that was needed in the current experiments to monitor stable pyruvate excretion. Uncoupling agents affect not only the investigated transporter, but in the long run the complete metabolism, making a clear statement on the transport mechanism problematic. Thus, the setup of the pyruvate export assay has to be modified first to enable quicker transport analysis. Preloading of cells with ^{14}C -glucose with the concomitant inhibition of PDH either by AEP or by deletion of its E1 subunit, as in the $\Delta aceE$ strain, should result in pyruvate accumulation and subsequent efflux. Transport inhibiting agents would cause higher pyruvate accumulation upon inhibition of the exporter, which can be then monitored by the accumulation of the radioactive label within the cells. This would allow the characterization of the driving force that the transporter relies on. Furthermore, these experiments might indicate whether *C. glutamicum* has only one pyruvate exporter or whether several transporters can mediate its efflux. Work on the isolated protein or – if applicable – with inverted membrane vesicles would enable accurate kinetic measurements, so that the kinetic parameters can be derived. Furthermore, experiments regarding the transporter specificity could be carried out then without the need of their intracellular accumulation.

In addition, the analysis of the transporter regulation regarding the transcription factors involved would be beneficial for further understanding of the transporter function and its entanglement in the cell's metabolism. A broad metabolome analysis might reveal further regulatory aspects of carboxylate metabolism in addition to the transcriptome assay. The accumulation of a certain substrate might indicate reduced activity of the enzyme converting it. Thus, further insights on the regulatory and metabolic network of carboxylate metabolism can be gained.

All in all, the presented work clarified the mechanisms involved in the uptake of carboxylates of the central metabolism. The knowledge gained in this work could be instantly utilized for the optimization of biotechnological production processes either by the expression of the respective transport system in order to enable or to improve the uptake of mono- and dicarboxylates by *C. glutamicum* or by their deletion to avoid re-uptake of the desired product. The possible control over carboxylate uptake is a decisive step for the construction of production strains. In order to introduce *C. glutamicum* into large-scale carboxylate biosynthesis, the identification of carboxylate export systems is required. Therefore, further work should be aimed at that direction.

5 Summary

This work focused on carboxylate transport in *C. glutamicum*. Substrate import and the export of compounds determine the metabolism and are therefore important factors to consider for correct estimation of fluxes. The presence of transport systems is mandatory, since most of the carboxylates analyzed in this work were shown to be membrane impermeable. The knowledge on the involved systems is of crucial importance for biotechnological applications. In this work, a suggested pyruvate importer was analyzed in detail and two further carboxylate importers were identified. Several different approaches were undertaken in order to identify carboxylate exporters.

The import of monocarboxylates such as pyruvate, acetate, and propionate was shown to be mediated by MctC in a proton dependent manner. MctC proved to be indispensable for pyruvate utilization and is also the only importer for acetate and propionate. The deletion of *mctC* enabled the differentiation of active and passive acetate/propionate influx, and the quantification of the contribution of MctC and diffusion to substrate uptake. Nevertheless, MctC was shown to have higher affinity for the membrane permeable substrates than for the impermeable substrate pyruvate. Its presence was demonstrated to confer an advantage at alkaline conditions and low concentrations of acetate. MctC activity was found to be dependent on the presence of pyruvate in the medium and to be regulated by the major regulators of acetate metabolism RamA and RamB. Furthermore, *mctC* was shown to be transcribed in an operon with a gene encoding a small membrane protein. Because of the operon structure and proton dependence of further SSS-type transporters, a new subgroup within this class was proposed.

Dicarboxylates were shown to be transported by two different secondary active transporters, which were identified in this work. DccT was shown to import C₄-dicarboxylates in dependence on Na⁺. DctA was shown to be H⁺ dependent and to have a broad substrate spectrum. In addition to C₄-dicarboxylates, indications for transport of the C₆-tricarboxylate citrate, the C₅-dicarboxylate 2-oxoglutarate, aspartate, and the monocarboxylates glyoxylate and lactate via DctA were given. Although it showed a broader substrate spectrum than DccT, its substrate affinity was found to be lower. Although both genes are present in *C. glutamicum*, they did not promote aerobic growth on these substrates unless they were artificially expressed or their expression was increased due to a mutation in the promoter region.

In regard to substrate efflux at microaerobic conditions, *C. glutamicum* ATCC 13032 was found to excrete mostly lactate, but also minor amounts of succinate and acetate. Site-directed mutagenesis indicated the involvement of Cgl2211 in succinate export, since the affected mutant was shown to excrete less of the carboxylates and to accumulate succinate

at oxygen deprived conditions. Although many different experiments were carried out in order to identify the lactate exporter, none of them was successful, indicating the presence of several lactate exporters in this organism.

The efflux of pyruvate can be triggered by the inhibition of pyruvate dehydrogenase, which was shown to influence the transcription rate of several genes encoding enzymes involved in major pathways using pyruvate as a precursor. This transcriptomics approach and phenotypic analysis of site-directed mutants revealed several putative pyruvate exporter candidates. The deletion of the respective genes was found to cause reduced pyruvate accumulation in the medium. Further biochemical characterization will address their actual participation in pyruvate export.

The achievements towards the clarification of carboxylate uptake and excretion presented here might substantially promote the exploitation of *C. glutamicum* in biotechnological applications.

6 References

- Abe, S., Takayama, K., Kinoshita, S. (1967)** Taxonomical studies on glutamic acid producing bacteria. *J. Gen. Microbiol.* 13: 279-301.
- Abe, K., Hayashi, H., Maloney, P.C. (1996)** Exchange of aspartate and alanine. Mechanism for development of a proton-motive force in bacteria. *J. Biol Chem.* 271(6):3079-84.
- Abe, K., Ohnishi, F., Yagi, K., Nakajima, T., Higuchi, T., Sano, M., Machida, M., Sarker, R.I., Maloney, P.C. (2002)** Plasmid-encoded *asp* operon confers a proton motive metabolic cycle catalyzed by an aspartate-alanine exchange reaction. *J Bacteriol.* 184(11):2906-13.
- Arndt, A., Auchter, M., Ishige, T., Wendisch, V.F., Eikmanns, B.J. (2007)** Ethanol catabolism in *Corynebacterium glutamicum*. *J. Mol. Microbiol. Biotechnol.* (Epub ahead of print).
- Arndt, A., and Eikmanns, B.J. (2007)** The alcohol dehydrogenase gene *adhA* in *Corynebacterium glutamicum* is subject to carbon catabolite repression. *J. Bacteriol.* 189(20):7408-16.
- Arndt, A. and Eikmanns, B.J. (2008)** Regulation of carbon metabolism in *Corynebacterium glutamicum*. In *Corynebacteria: Genomics and molecular biology* (Burkovski A., ed.), Horizon Scientific Press, Norwich, UK, pp. 155-182.
- Asai, K., Baik, S.H., Kasahara, Y., Moriya, S., Ogasawara, N. (2000)** Regulation of the transport system for C₄-dicarboxylic acids in *Bacillus subtilis*. *Microbiology.* 146:263-71.
- Baker, K.E., Ditullio, K.P., Neuhaud, J., Kelln, R.A. (1996)** Utilization of orotate as a pyrimidine source by *Salmonella typhimurium* and *Escherichia coli* requires the dicarboxylate transport protein encoded by *dctA*. *J. Bacteriol.* 178(24):7099-105.
- Ballan, S. (2007)** Untersuchung zum Transport von Acetat und Pyruvat in *Corynebacterium glutamicum*. Diploma thesis, University of Cologne.
- Bandell, M., Lhotte, M.E., Marty-Teyssset, C., Veyrat, A., Prévost, H., Dartois, V., Diviès, C., Konings, W.N., Lolkema, J.S. (1998)** Mechanism of the citrate transporters in carbohydrate and citrate cometabolism in *Lactococcus* and *Leuconostoc* species. *Appl. Environ. Microbiol.* 64(5):1594-600.
- Baumbach, J., Brinkrolf, K., Czaja, L.F., Rahmann, S., Tauch, A. (2006)** CoryneRegNet: an ontology-based data warehouse of corynebacterial transcription factors and regulatory networks. *BMC Genomics.* 14:7-24.
- Becker, M. (2007)** Untersuchungen zum Kaliumtransport in *Corynebacterium glutamicum*. Diplomarbeit, Universität zu Köln Diploma thesis, University of Cologne.

- Berovic, M., and Legisa, M. (2007)** Citric acid production. *Biotechnol. Annu. Rev.* 13:303-43
- Blombach, B., Schreiner, M.E., Holatko, J., Bartek, T., Oldiges, M., Eikmanns, B.J. (2007a)** L-valine production with pyruvate dehydrogenase complex-deficient *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 73(7):2079-84.
- Blombach, B., Schreiner, M.E., Moch, M., Oldiges, M., Eikmanns, B.J. (2007b)** Effect of pyruvate dehydrogenase complex deficiency on L-lysine production with *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* 76:615-23.
- Bott, M., Meyer, M., Dimroth, P. (1995)** Regulation of anaerobic citrate metabolism in *Klebsiella pneumoniae*. *Mol. Microbiol.* 18(3):533-46.
- Bott, M. and Niebisch, A. (2005)** Respiratory energy metabolism. In *Handbook of Corynebacterium glutamicum*, (Eggeling, L. and Bott, M., eds), pp. 305-332, CRC Press, Boca Raton, Florida, USA.
- Bott, M. (2007)** Offering surprises: TCA cycle regulation in *Corynebacterium glutamicum*. *Trends Microbiol.* 15(9):417-25.
- Bradford, M.M. (1976)** A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Brinkrolf, K., Brune, I., Tauch, A. (2006)** Transcriptional regulation of catabolic pathways for aromatic compounds in *Corynebacterium glutamicum*. *Genet. Mol. Res.* 7;5(4):773-89.
- Brinkrolf, K., Plöger, S., Solle, S., Brune, I., Nentwich, S.S., Hüser, A.T., Kalinowski, J., Pühler, A., Tauch, A. (2008)** The LacI/GalR family transcriptional regulator UriR negatively controls uridine utilization of *Corynebacterium glutamicum* by binding to catabolite-responsive element (cre)-like sequences. *Microbiology.* 154(Pt 4):1068-81.
- Brock, M. and Buckel, W. (2004)** On the mechanism of action of the antifungal agent propionate. *Eur. J. Biochem.* 271(15):3227-41.
- Brune, I., Werner, H., Hüser, A.T., Kalinowski, J., Pühler, A., Tauch, A. (2006)** The DtxR protein acting as dual transcriptional regulator directs a global regulatory network involved in iron metabolism of *Corynebacterium glutamicum*. *BMC Genomics.* 9;7:21.
- Busch, A.E., Waldegger, S., Herzer, T., Biber, J., Markovich, D., Murer, H., Lang, F. (1994)** Electrogenic cotransport of Na⁺ and sulfate in *Xenopus* oocytes expressing the cloned Na⁺SO₄⁽²⁻⁾ transport protein NaSi-1. *J. Biol. Chem.* 269(17):12407-9.
- Busch, W., and M. H. Saier, Jr. (2004)** The IUBMB-endorsed transporter classification system. *Mol. Biotechnol.* 27:253-62.
- Causey, T.B., Zhou, S., Shanmugam, K.T., Ingram, L.O. (2003)** Engineering the metabolism of *Escherichia coli* W3110 for the conversion of sugar to redox-neutral and oxidized products: homoacetate production. *Proc. Natl. Acad. Sci. U S A.* 100(3):825-32.

- Chaudhry, M.T., Huang, Y., Shen, X.H., Poetsch, A., Jiang, C.Y., Liu, S.J. (2007)** Genome-wide investigation of aromatic acid transporters in *Corynebacterium glutamicum*. Microbiology. 153(Pt 3):857-65.
- Claes, W.A., Pühler, A., Kalinowski, J. (2002)** Identification of two *prpDBC* gene clusters in *Corynebacterium glutamicum* and their involvement in propionate degradation via the 2-methylcitrate cycle. J. Bacteriol. 184(10):2728-39.
- Cocaign, M., Monnet, C., Lindley, N.D. (1993)** Batch kinetics of *Corynebacterium glutamicum* during growth on various substrates: use of substrate mixtures to localise metabolic bottlenecks. Appl. Microbiol. Biotechnol. 40:526–530.
- Cramer, A., Gerstmeir, R., Schaffer, S., Bott, M., Eikmanns, B.J. (2006)** Identification of RamA, a novel LuxR-type transcriptional regulator of genes involved in acetate metabolism of *Corynebacterium glutamicum*. J. Bacteriol. 188(7):2554-67.
- Cramer, A., Auchter, M., Frunzke, J., Bott, M., Eikmanns B.J. (2007)** RamB, the transcriptional regulator of acetate metabolism in *Corynebacterium glutamicum*, is subject to regulation by RamA and RamB. J. Bacteriol. 189:1145-9.
- Crolla, A. and Kennedy, K.J. (2004)** Fed-batch production of citric acid by *Candida lipolytica* grown on *n*-paraffins. J. Bacteriol. 110(1):73-84.
- Dien, B.S., Nichols, N.N., Bothast, R.J. (2002)** Fermentation of sugar mixtures using *Escherichia coli* catabolite repression mutants engineered for production of L-lactic acid. J. Ind. Microbiol. Biotechnol. 29(5):221-7.
- Dominguez, H., Rollin, C., Guyonvarch, A., Guerquin-Kern, J.L., Cocaign-Bousquet, M., Lindley, N.D. (1998)** Carbon-flux distribution in the central metabolic pathways of *Corynebacterium glutamicum* during growth on fructose. Eur. J. Biochem. 254(1):96-102.
- Ebbighausen, H., Weil, B., Krämer, R. (1991a)** Carrier mediated acetate uptake in *Corynebacterium glutamicum*. Arch. Microbiol. 155, 505-510.
- Ebbighausen, H., Weil, B., Krämer. R. (1991b)** Na⁺-dependent succinate uptake in *Corynebacterium glutamicum*. FEMS Microbiol. Lett. 61:61-5.
- Eikmanns, B.J., Thum-Schmitz, N., Eggeling, L., Lüdtkke, K.U., Sahm, H. (1994)** Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum* *gltA* gene encoding citrate synthase. Microbiology. 140 (Pt 8):1817-28.
- Eikmanns, B. (2005)** Central metabolism: Tricarboxylic Acid Cycle and anaplerotic reactions. In Handbook of *Corynebacterium glutamicum*, (Eggeling, L. and Bott, M., eds), pp. 241-276, CRC Press, Boca Raton, Florida, USA.
- Engel, P., Krämer, R., Unden, G. (1992)** Anaerobic fumarate transport in *Escherichia coli* by an *fnr*-dependent dicarboxylate uptake system which is different from the aerobic dicarboxylate uptake system. J. Bacteriol. 174(17):5533-9.

- Engel, P., Krämer, R., Uden, G. (1994)** Transport of C₄-dicarboxylates by anaerobically grown *Escherichia coli*. Energetics and mechanism of exchange, uptake and efflux. Eur. J. Biochem. 222(2):605-14.
- Engels, V., and Wendisch, V.F. (2007)** The DeoR-type regulator SugR represses expression of *ptsG* in *Corynebacterium glutamicum*. J. Bacteriol. 189(8):2955-66.
- Fiur, D. (2004)** Charakterisierung des Pyruvatdehydrogenase-Komplexes aus *Corynebacterium glutamicum* und Einfluss von Enzymen des anaplerotischen Knotenpunkts auf die Bildung von Valin und Pantothenat. Dissertation, University of Ulm.
- Follmann, M. (2004)** Aufnahme von Methionin in *Corynebacterium glutamicum*: Identifizierung und Charakterisierung der beteiligten Transportproteine. Diploma thesis, University of Cologne.
- Follmann, M. (2008)** Untersuchungen zum Einfluss von pH-Variation und erhöhter CO₂ Konzentration auf Stoffwechsel und Aminosäureproduktion mit *Corynebacterium glutamicum*. Dissertation, Universität zu Köln.
- Forward, J.A., Behrendt, M.C., Wyborn, N.R., Cross, R., Kelly, D.J. (1997)** TRAP transporters: a new family of periplasmic solute transport systems encoded by the *dctPQM* genes of *Rhodobacter capsulatus* and by homologs in diverse gram-negative bacteria. J. Bacteriol. 179(17):5482-93.
- Frunzke, J., Engels, V., Hasenbein, S., Gätgens, C., Bott, M. (2008)** Coordinated regulation of gluconate catabolism and glucose uptake in *Corynebacterium glutamicum* by two functionally equivalent transcriptional regulators, GntR1 and GntR2. Mol. Microbiol. 67: 305–322
- Fudou, R., Jojima, Y., Seto, A., Yamada, K., Rimura, E., Nakamatsu, T., Hirashi, A., Yamanaka, S. (2002)** *Corynebacterium efficiens* sp. nov., a glutamic-acid-producing species from soil and plant material. Int. J. Syst. Evol. Microbiol. 52, 1127-1131.
- Georgi, T., Engels, V., Wendisch, V.F. (2008)** Regulation of L-lactate utilization by the FadR-type regulator LldR of *Corynebacterium glutamicum*. J. Bacteriol. 190(3):963-71.
- Gerstmeir, R., Wendisch, V.F., Schnicke, S., Ruan, H., Farwick, M., Reinscheid, D., Eikmanns, B.J. (2003)** Acetate metabolism and its regulation in *Corynebacterium glutamicum*. J. Biotechnol. 104(1-3):99-122.
- Gerstmeir, R., Cramer, A., Dangel, P., Schaffer, S., Eikmanns, B. J. (2004)** RamB, a novel transcriptional regulator of genes involved in acetate metabolism of *Corynebacterium glutamicum*. J. Bacteriol. 186:2798-809.
- Gimenez, R., Nuñez, M.F., Badia, J., Aguilar, J., Baldoma, L. (2003)** The gene *yjcG*, cotranscribed with the gene *acs*, encodes an acetate permease in *Escherichia coli*. J. Bacteriol. 185(21):6448-55.

- Golby, P., Kelly, D.J., Guest, J.R., Andrews, S.C. (1998)** Transcriptional regulation and organization of the *dcuA* and *dcuB* genes, encoding homologous anaerobic C₄-dicarboxylate transporters in *Escherichia coli*. J. Bacteriol. 180(24):6586-96.
- Grant, S.G.N., Jessee, J., Bloom, F.R., Hanahan, D. (1990)** Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad., USA, 87: 4645-4649.
- Gutowski, S. J., and H. Rosenberg. (1975)** Succinate uptake and related proton movements in *Escherichia coli* K-12. Biochem. J. 152:647–654.
- Hagmann, C. (2007)** Glykogensynthese in *Corynebacterium glutamicum* – Regulation des *glgC*-Gens. Diploma thesis, University of Ulm.
- Halestrap, A.P., Price, N.T. (1999)** The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochem. J. 343 (Pt 2) 281-99.
- Halgasova, N., Bukovska, G., Timko, J., Kormanec, J. (2001)** Cloning and transcriptional characterization of two sigma factor genes, *sigA* and *sigB*, from *Brevibacterium flavum*. Curr. Microbiol. 43, 249-254.
- Hall, J.A., and Pajor, A.M. (2005)** Functional characterization of a Na⁺-coupled dicarboxylate carrier protein from *Staphylococcus aureus*. J. Bacteriol. 187(15):5189-94.
- Han, S.O., Inui, M., Yukawa, H. (2007)** Expression of *Corynebacterium glutamicum* glycolytic genes varies with carbon source and growth phase. Microbiology 153: 2190-2202.
- Hatti-Kaul, R., Törnvall, U., Gustafsson, L., Börjesson, P. (2007)** Industrial biotechnology for the production of bio-based chemicals - a cradle-to-grave perspective. Trends Biotechnol. 25(3):119-24.
- Hayashi, M., Mizoguchi, H., Shiraishi, N., Obayashi, M., Nakagawa, S., Imai, J., Watanabe, S., Ota, T., Ikeda, M. (2002)** Transcriptome analysis of acetate metabolism in *Corynebacterium glutamicum* using a newly developed metabolic array. Biosci. Biotechnol. Biochem. 66(6):1337-44.
- Helmann, J.D. (2002)** The extracytoplasmic function (ECF) sigma factors. Adv. Microb. Physiol. 46, 47-110.
- Hermann, T. (2003)** Industrial production of amino acids by coryneform bacteria. J. Biotechnol. 104: 155-172
- Hobman, J.L. (2007)** MerR family transcription activators: similar designs, different specificities. Mol. Microbiol. 63(5):1275-8.
- Horswill, A.R., Dudding, A.R., Escalante-Semerena, J.C. (2001)** Studies of propionate toxicity in *Salmonella enterica* identify 2-methylcitrate as a potent inhibitor of cell growth. J Biol Chem 276: 19094–19101.

- Hosie, A.H., Allaway, D., Poole, P.S. (2002)** A monocarboxylate permease of *Rhizobium leguminosarum* is the first member of a new subfamily of transporters. *J. Bacteriol.* 184(19):5436-48.
- Hüser, A.T., Becker, A., Brune, I., Dondrup, M., Kalinowski, J., Plassmeier, J., Pühler, A., Wiegräbe, I., Tauch, A. (2003)** Development of a *Corynebacterium glutamicum* DNA microarray and validation by genome-wide expression profiling during growth with propionate as carbon source. *J. Biotechnol.* 106(2-3):269-86.
- Ikeda, M., and Nakagawa, S. (2003)** The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. *Appl. Microbiol. Biotechnol.* 62, 99-109.
- Inui, M., Murakami, S., Okino, S., Kawaguchi, H., Vertes, A.A., Yukawa, H. (2004)** Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. *J. Mol. Microbiol. Biotechnol.* 7(4):182-96.
- Inui, M., Suda, M., Okino, S., Nonaka, H., Puskás, L.G., Vertès, A.A, Yukawa, H. (2007)** Transcriptional profiling of *Corynebacterium glutamicum* metabolism during organic acid production under oxygen deprivation conditions. *Microbiology.* 153(Pt 8):2491-504.
- Ito, M., Guffanti, A.A., Oudega, B., Krulwich, T.A. (1999)** Mrp, a multigene, multifunctional locus in *Bacillus subtilis* with roles in resistance to cholate and to Na⁺ and in pH homeostasis. *J. Bacteriol.* 181: 2394-2402.
- Ito, M., Guffanti, A.A., Wang, W., Krulwich, T.A. (2000)** Results of non-polar mutations in each of the seven *Bacillus subtilis* mrp genes suggest complex interactions among the gene products in support of Na⁺- and Alkali- but not cholate-resistance. *J. Bacteriol.* 182: 5663-5670.
- Janausch, I.G., Kim, O.B., Unden, G. (2001)** DctA- and Dcu-independent transport of succinate in *Escherichia coli*: contribution of diffusion and of alternative carriers. *Arch. Microbiol.* 176(3):224-30.
- Janausch, I.G., Zientz, E., Tran, Q.H., Kröger, A., Unden, G. (2002)** C₄-dicarboxylate carriers and sensors in bacteria. *Biochim. Biophys. Acta.* 1553(1-2):39-56.
- Jetten, M.S.M. and A.J. Sinskey. (1993)** Characterization of phosphoenol pyruvate carboxykinase from *Corynebacterium glutamicum*. *FEMS Microbiol. Lett.* 111:183-188.
- Jetten, M.S., Gubler, M.E., Lee, S.H., Sinskey, A.J. (1994)** Structural and functional analysis of pyruvate kinase from *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 60(7):2501-7.
- Jolkver, E., Emer, D., Ballan, S., Krämer, R., Eikmanns, B., Marin, K. (2008)** Identification and characterization of a bacterial carrier system for the uptake of pyruvate, propionate and acetate in *Corynebacterium glutamicum*. Submitted.
- Kaim, G., and Dimroth, P. (1999)** ATP synthesis by F-type ATP synthase is obligatorily dependent on the transmembrane voltage. *EMBO J.* 18(15):4118-27.

- Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., Dusch, N., Eggeling, L., Eikmanns, B.J., Gaigalat, L., Goesmann, A., Hartmann, M., Huthmacher, K., Krämer, R., Linke, B., McHardy, A.C., Meyer, F., Möckel, B., Pfefferle, W., Pühler, A., Rey, D.A., Rückert, C., Rupp, O., Sahm, H., Wendisch, V.F., Wiegräbe, I., Tauch, A. (2003) The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J. Biotechnol. 104(1-3):5-25.
- Kase, H. and Nakayama, K. (1972) Production of L-threonine by analog-resistant mutants. Agric. Biol. Chem. 36: 1611-1621.
- Kästner, C.N., Schneider, K., Dimroth, P., Pos, K.M. (2002) Characterization of the citrate/acetate antiporter CitW of *Klebsiella pneumoniae*. Arch Microbiol. 177(6):500-6.
- Kay, W.W., and Kornberg, H.L. (1971) The uptake of C₄-dicarboxylic acids by *Escherichia coli*. Eur. J. Biochem. 18(2):274–281.
- Kell, D.B., Peck, M.W., Rodger, G., Morris, J.G. (1981) On the permeability to weak acids and bases of the cytoplasmic membrane of *Clostridium pasteurianum*. Biochem. Biophys. Res. Comm. 99, 81-88
- Kelle, R., Hermann, T., Bathe, B. (2005) L-Lysine production. In Handbook of *Corynebacterium glutamicum*, (Eggeling, L. and Bott, M., eds), pp. 465-488, CRC Press, Boca Raton, Florida, USA
- Kelly, D.J. and Thomas, G.H. (2001) The tripartite ATP-independent periplasmic (TRAP) transporters of bacteria and archaea. FEMS Microbiol. Rev. 25(4):405-24.
- Kennerknecht, N., Sahm, H., Yen, M.R., Patek, M., Saier, M.H. Jr, Eggeling, L. (2002) Export of L-isoleucine from *Corynebacterium glutamicum*: a two-gene-encoded member of a new translocator family. J. Bacteriol. 184(14):3947-56.
- Kim, H.J., Kim, T.H., Kim, Y., Lee, H.S. (2004) Identification and characterization of *glxR*, a gene involved in regulation of glyoxylate bypass in *Corynebacterium glutamicum*. J. Bacteriol. 186(11):3453-60.
- Kimata, K., Tanaka, Y., Inada, T., Aiba, H. (2001) Expression of the glucose transporter gene, *ptsG*, is regulated at the mRNA degradation step in response to glycolytic flux in *Escherichia coli*. EMBO J. 20(13):3587-95.
- Kimura, E. (2002) Triggering mechanism of L-glutamate overproduction by DtsR1 in Coryneform Bacteria. J. Biosci. Bioeng. 94, 545-551.
- Kinoshita, S., Shigezo, U., Shimono, M. (1957) Studies on the amino acid fermentation: Part I: Production of L-glutamic acid by various microorganisms. J. Gen. Appl. Microbiol. 3, 193-205.
- Kocan, M., Schaffer, S., Ishige T., Sorger-Hermann, U., Wendisch, V. F., Bott, M. (2006) Two-component systems of *Corynebacterium glutamicum*: deletion analysis and

- involvement of the PhoS-PhoR system in the phosphate starvation response. J. Bacteriol. 188: 724-732.
- Kohl, T.A., Baumbach, J., Jungwirth, B., Pühler, A., Tauch, A. (2008)** The GlxR regulon of the amino acid producer *Corynebacterium glutamicum*: *In silico* and *in vitro* detection of DNA binding sites of a global transcription regulator. J Biotechnol. 135(4):340-350.
- Krämer, R., Lambert, C., Hoischen, C., Ebbighausen, H. (1990)** Uptake of glutamate in *Corynebacterium glutamicum*. 1. Kinetic properties and regulation by internal pH and potassium. Eur. J. Biochem. 194(3):929-35.
- Krämer, R. (1999)** Multiple roles of prokaryotic cell membranes. In Biology of the Prokaryotes. (Lengeler, J.W., Drews, G., Schlegel, H.G., eds.). London: Blackwell Science Inc.
- Krogh, A., Larsson, B., von Heijne, G., Sonnhammer, E.L. (2001)** Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305, 567-580.
- Krömer, J.O., Sorgenfrei, O., Klopprogge, K., Heinzle, E., Wittmann, C. (2004)** In-depth profiling of lysine-producing *Corynebacterium glutamicum* by combined analysis of the transcriptome, metabolome, and fluxome. J. Bacteriol. 186(6):1769-84.
- Kronmeyer, W., Peekhaus, N., Krämer, R., Sahm, H., Eggeling, L. (1995)** Structure of the *gluABCD* cluster encoding the glutamate uptake system of *Corynebacterium glutamicum*. J. Bacteriol. 177(5):1152-8.
- Krug, A., Wendisch, V.F., Bott, M. (2005)** Identification of AcnR, a TetR-type repressor of the aconitase gene *acn* in *Corynebacterium glutamicum*. J. Biol. Chem. 280(1):585-95.
- Laber, B., and Amrhein, N. (1987)** Metabolism of 1-aminoethylphosphinate generates acetylphosphinate, a potent inhibitor of pyruvate dehydrogenase. Biochem. J. 248(2):351–358.
- Larisch, C., Nakunst, D., Hüser, A.T., Tauch, A., Kalinowski, J. (2007)** The alternative sigma factor SigB of *Corynebacterium glutamicum* modulates global gene expression during transition from exponential growth to stationary phase. BMC Genomics 8, 4.
- Leuchtenberger, W. (1996)** Amino acids-technical production and use. In Products of primary metabolism. (Rehm, H. and Reed, G., eds). Biotechnology. VCH Weinheim, pp. 455-502.
- Li, Y., Chen, J., Lun, S.Y. (2001)** Biotechnological production of pyruvic acid. Appl. Microbiol. Biotechnol. 57(4):451-9.
- Liebl, W. (2001)** *Corynebacterium* nonmedical. In The Prokaryotes. An Evolving Electronic Resource for the Microbiological Community, (M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt, eds.), New-York, USA, Springer, pp. 796-818.

- Lin, H., Bennett, G.N., San, K.Y. (2005)** Fed-batch culture of a metabolically engineered *Escherichia coli* strain designed for high-level succinate production and yield under aerobic conditions. *Biotechnol. Bioeng.* 90(6):775-9.
- Maas, R.H., Bakker, R.R., Eggink, G., Weusthuis, R.A. (2006)** Lactic acid production from xylose by the fungus *Rhizopus oryzae*. *Appl. Microbiol. Biotechnol.* 72(5):861-8.
- Makarova, K.S., Mironov, A.A., Gelfand, M.S. (2001)** Conservation of the binding site for the arginine repressor in all bacterial lineages. *Genome Biol.* 2(4).
- Maklashina, E., Berthold, D.A., Cecchini, G. (1998)** Anaerobic expression of *Escherichia coli* succinate dehydrogenase: functional replacement of fumarate reductase in the respiratory chain during anaerobic growth. *J. Bacteriol.* 180(22):5989-96.
- Marin, K., and Krämer, R. (2007)** Amino acid transport systems in biotechnologically relevant bacteria. In *Amino Acid Biosynthesis – Pathways, Regulation and Metabolic Engineering*, Vol. 5, (Wendisch, V. ed.), series: Microbiology Monographs, (Steinbüchel, A. ed.)
- Markovich, D., and Murer, H. (2004)** The SLC13 gene family of sodium sulphate/carboxylate cotransporters. *Pflugers Arch.* 447(5):594-602
- Marty-Teyssset, C., Lolkema, J.S., Schmitt, P., Divies, C., Konings, W.N. (1995)** Membrane potential-generating transport of citrate and malate catalyzed by CitP of *Leuconostoc mesenteroides*. *J. Biol. Chem.* 270(43):25370-6.
- Maruyama, K., and Kitamura, H. (1985)** Mechanisms of growth inhibition by propionate and restoration of the growth by sodium bicarbonate or acetate in *Rhodopseudomonas sphaeroides* S. *J. Biochem.* 98(3):819-24.
- Marx, A., Hans, S., Möckel, B., Bathe, B., de Graaf, A.A., McCormack, A.C., Stapleton, C., Burke, K., O'Donohue, M., Dunican, L.K. (2003)** Metabolic phenotype of phosphoglucose isomerase mutants of *Corynebacterium glutamicum*. *J. Biotechnol.* 104(1-3):185-97.
- Mateos, L.M., Ordóñez, E., Letek, M., Gil, J.A. (2006)** *Corynebacterium glutamicum* as a model bacterium for the bioremediation of arsenic. *Int. Microbiol.* 9(3):207-15.
- Matsushita, K., Inoue, T., Adachi, O., Toyama, H. (2005)** *Acetobacter aceti* possesses a proton motive force-dependent efflux system for acetic acid. *J. Bacteriol.* 187(13):4346-52.
- Mauzy, C.A., and Hermodson, M.A. (1992)** Structural and functional analyses of the repressor, RbsR, of the ribose operon of *Escherichia coli*. *Protein Sci.* 1(7):831-42.
- Menkel, E., Thierbach, G., Eggeling, L., Sahm, H. (1989)** Influence of increased aspartate availability on lysine formation by a recombinant strain of *Corynebacterium glutamicum* and utilization of fumarate. *Appl. Environ. Microbiol.* 55(3):684-8.
- Mohrbach, T. (2006)** Untersuchungen zum Import und Export von Tryptophan in *Corynebacterium glutamicum*. Diploma thesis, University of Cologne.

- Möker, N. (2006)** Sensing properties of MtrB-MtrA of *Corynebacterium glutamicum*: a twocomponent system involved in the osmo- and chill stress response, PhD thesis, University of Cologne.
- Möker, N., Bocker, M., Schaffner, S., Krämer, R., Morbach, S., Bott, M. (2004)** Deletion of two genes encoding the MtrA-MtrB two-component system of *Corynebacterium glutamicum* has strong influence on cell morphology, antibiotics susceptibility and expression of genes involved in osmoprotection, Mol. Microbiol. 54, 420-438.
- Molenaar, D., van der Rest, M.E., Drysch, A., Yücel, R. (2000)** Functions of the membrane-associated and cytoplasmic malate dehydrogenases in the citric acid cycle of *Corynebacterium glutamicum*. J. Bacteriol. 182(24):6884-91.
- Moon, M.W., Kim, H.J., Oh, T.K., Shin, C.S., Lee, J.S., Kim, S.J., Lee, J.K. (2005)** Analyses of enzyme II gene mutants for sugar transport and heterologous expression of fructokinase gene in *Corynebacterium glutamicum* ATCC 13032. FEMS Microbiol. Lett. 244(2):259-66.
- Moon, M.W., Park, S.Y., Choi, S.K., Lee, J.K. (2007)** The phosphotransferase system of *Corynebacterium glutamicum*: features of sugar transport and carbon regulation. J. Mol. Microbiol. Biotechnol. 12(1-2):43-50.
- Mormann, S., Lomker, A., Ruckert, C., Gaigalat, L., Tauch, A., Pühler, A., Kalinowski, J. (2006)** Random mutagenesis in *Corynebacterium glutamicum* ATCC 13032 using an IS6100-based transposon vector identified the last unknown gene in the histidine biosynthesis pathway. BMC Genomics. 7:205.
- Muffler, A., Bettermann, S., Haushalter, M., Hörlein, A., Neveling, U., Schramm, M., Sorgenfrei, O. (2002)** Genome-wide transcription profiling of *Corynebacterium glutamicum* after heat shock and during growth on acetate and glucose. J. Biotechnol. 98(2-3):255-68.
- Mullis, K. B., Farone, F. A., Schar, S., Saiki, R., Horn, G., and Ehrlich, H. (1986)** Specific amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. 51: 263-273.
- Nakano, S., Fukaya, M., Horinouchi, S. (2006)** Putative ABC transporter responsible for acetic acid resistance in *Acetobacter aceti*. Appl. Environ. Microbiol. 72(1):497-505.
- Nakunst, D., Larisch, C., Hüser, A.T., Tauch, A., Pühler, A., Kalinowski, J. (2007)** The extracytoplasmic function-type sigma factor SigM of *Corynebacterium glutamicum* ATCC 13032 is involved in transcription of disulfide stress-related genes. J. Bacteriol. 189(13):4696-707.
- Nanatani, K., T. Fujiki, K. Kanou, M. Takeda-Shitaka, H. Umeyama, L. Ye, X. Wang, T. Nakajima, T. Uchida, P.C. Maloney, Abe, K. (2007)** Topology of AspT, the aspartate:alanine antiporter of *Tetragenococcus halophilus*, determined by site-directed fluorescence labeling. J. Bacteriol. 189: 7089-7097.

- Nesvera, J., and Patek, M. (2008)** Plasmids and promoters in *Corynebacteria* and their applications. In *Corynebacteria: Genomics and molecular biology* (Burkovski A., ed.), Horizon Scientific Press, Norwich, UK, pp. 113-155.
- Netzer, R., Peters-Wendisch, P., Eggeling, L., and Sahm, H. (2004)** Cometabolism of a nongrowth substrate: L-serine utilization by *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 70: 7148-7155.
- Niebisch, A. and Bott, M. (2001)** Molecular analysis of the cytochrome bc1-aa3 branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome c1. *Arch. Microbiol.* 175: 282-294.
- Niebisch, A., Kabus, A., Schultz, C., Weil, B., Bott, M. (2006)** Corynebacterial protein kinase G controls 2-oxoglutarate dehydrogenase activity via the phosphorylation status of the OdhI protein. *J. Biol. Chem.* 281(18):12300-7.
- Nies, D.H. (2003)** Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol. Rev.* 27(2-3):313-39.
- Nishimura, T., Vertès, A.A., Shinoda, Y., Inui, M., Yukawa, H. (2007)** Anaerobic growth of *Corynebacterium glutamicum* using nitrate as a terminal electron acceptor. *Appl. Microbiol. Biotechnol.* 75(4):889-97.
- Nisonoff, A., Barnes, F.W. Jr., Enns, T. (1953)** Mechanisms in enzymatic transamination; estimation of velocity of the glutamate-aspartate reaction at equilibrium. *J. Biol. Chem.* 204, 957-969
- Nixon, B.T, Ronson, C.W., Ausubel, F.M. (1986)** Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with nitrogen assimilation regulatory genes *ntrB* and *ntrC*. *Proc. Natl. Acad. Sci. USA* 83: 7850-7854.
- Núñez, M.F., Kwon, O., Wilson, T.H., Aguilar, J., Baldoma, L., Lin, E.C. (2002)** Transport of L-Lactate, D-Lactate, and glycolate by the LldP and GlcA membrane carriers of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 290(2):824-9.
- Otto, R., Sonnenberg, A.S., Veldkamp, H., Konings, W.N. (1980)** Generation of an electrochemical proton gradient in *Streptococcus cremoris* by lactate efflux. *Proc. Natl. Acad. Sci. U S A.* 77(9):5502-6.
- Pajor, A.M. (1995)** Sequence and functional characterization of a renal sodium/dicarboxylate cotransporter. *J. Biol. Chem.* 270(11):5779-85.
- Palmieri, F. (1994)** Mitochondrial carrier proteins. *FEBS Lett.* 346(1):48-54.
- Papanikolaou, S., Komaitis, M., Aggelis, G. (2004)** Single cell oil (SCO) production by *Mortierella isabellina* grown on high-sugar content media. *Bioresour. Technol.* 95 (3):287-91.
- Pátek, M. (2005).** Regulation of Gene Expression. In *Handbook of Corynebacterium glutamicum*, (Eggeling, L. and Bott, M. eds), pp. 81-98, CRC Press, Boca Raton, Florida, USA.

- Peter, H., Bader, A., Burkovski, A., Lambert, C., Krämer, R. (1997)** Isolation of the putP gene of *Corynebacterium glutamicum* and characterization of a low-affinity uptake system for compatible solutes. Arch. Microbiol. 168(2):143-51.
- Petersen, S., de Graaf, A.A., Eggeling, L., Möllney, M., Wiechert, W., Sahm, H. (2000)** In vivo quantification of parallel and bidirectional fluxes in the anaplerosis of *Corynebacterium glutamicum*. J. Biol. Chem. 275(46):35932-41.
- Peters-Wendisch P.G., Schiel B., Wendisch V.F., Katsoulidis E., Möckel B., Sahm H., Eikmanns B.J. (2001)** Pyruvate carboxylase is a major bottleneck for glutamate and lysine production by *Corynebacterium glutamicum*. J. Mol. Microbiol. Biotechnol. 3(2):295-300.
- Plassmeier, J., Barsch, A., Persicke, M., Niehaus, K., Kalinowski, J. (2007)** Investigation of central carbon metabolism and the 2-methylcitrate cycle in *Corynebacterium glutamicum* by metabolic profiling using gas chromatography-mass spectrometry. J Biotechnol 130:354-63.
- Polen, T., Schluesener, D., Poetsch, A., Bott, M., Wendisch, V.F. (2007)** Characterization of citrate utilization in *Corynebacterium glutamicum* by transcriptome and proteome analysis. FEMS Microbiol. Lett. 273(1):109-19.
- Pos, K.M., Dimroth, P., Bott, M. (1998)** The *Escherichia coli* citrate carrier CitT: a member of a novel eubacterial transporter family related to the 2-oxoglutarate/malate translocator from spinach chloroplasts. J. Bacteriol. 180(16):4160-5.
- Price, N.T., Jackson, V.N., Halestrap, A.P. (1998)** Cloning and sequencing of four new mammalian monocarboxylate transporter (MCT) homologues confirms the existence of a transporter family with an ancient past. Biochem. J. 329 (Pt 2):321-8.
- Putnoky, P., A. Kereszt, T. Nakamura, G. Endre, E. Grosskopf, P. Kiss, Kondorosi, A. (1998)** The pha gene cluster of *Rhizobium meliloti* involved in pH adaptation and symbiosis encodes a novel type of K⁺ efflux system. Mol. Microbiol. 28: 1091-1101.
- Qi, S.W., Chaudhry, M.T., Zhang, Y., Meng, B., Huang, Y., Zhao, K.X., Pötsch, A., Jiang, C.Y., Liu, S., Liu, S.J. (2007)** Comparative proteomes of *Corynebacterium glutamicum* grown on aromatic compounds revealed novel proteins involved in aromatic degradation and a clear link between aromatic catabolism and gluconeogenesis via fructose-1,6-bisphosphatase. Proteomics. 7(20):3775-87.
- Quesada-Chanto, A., Afschar, A.S., Wagner, F. (1994)** Microbial production of propionic acid and vitamin B12 using molasses or sugar. Appl. Microbiol. Biotechnol. 41(4):378-83.
- Reddy, C.A., and Kao, M. (1978)** Value of acid metabolic products in identification of certain corynebacteria. J. Clin. Microbiol. 7(5):428-33.
- Reinscheid, D.J., Schnicke, S., Rittmann, D., Zahnow, U., Sahm, H., Eikmanns, B. J. (1999)** Cloning, sequence analysis, expression and inactivation of the *Corynebacterium*

- glutamicum pta-ack* operon encoding phosphotransacetylase and acetate kinase. Microbiology 145:503–513.
- Ren, Q., Chen, K., Paulsen, I.T. (2007)** TransportDB: a comprehensive database resource for cytoplasmic membrane transport systems and outer membrane channels. Nucleic Acids Res. 35.
- Rey, D.A., Nentwich, S.S., Koch, D.J., Rückert, C., Pühler, A., Tauch, A., Kalinowski, J. (2005)** The McbR repressor modulated by the effector substance S-adenosylhomocysteine controls directly the transcription of a regulon involved in sulphur metabolism of *Corynebacterium glutamicum* ATCC 13032. Mol. Microbiol. 56(4):871-87.
- Riedel, C., Rittmann, D., Dangel, P., Möckel, B., Petersen, S., Sahm, H., Eikmanns, B.J. (2001)** Characterization of the phosphoenolpyruvate carboxykinase gene from *Corynebacterium glutamicum* and significance of the enzyme for growth and amino acid production. J. Mol. Microbiol. Biotechnol. 3(4):573-83.
- Rittmann, D., Schaffer, S., Wendisch, V.F., Sahm, H. (2003)** Fructose-1,6-bisphosphatase from *Corynebacterium glutamicum*: expression and deletion of the *fbp* gene and biochemical characterization of the enzyme. Arch. Microbiol. 180(4):285-92.
- Roosild, T.P., Miller, S., Booth, I.R., Choe, S. (2002)** A mechanism of regulating transmembrane potassium flux through a ligand-mediated conformational switch. Cell 109(6):781-91.
- Rothfuss, F. and Conrad, R. (1993)** Vertical profiles of CH₄ concentrations, dissolved substrates, and processes involved in CH₄ production in a flooded Italian rice field. Biogeochemistry 18: 137-152
- Saitoh, S., Ishida, N., Onishi, T., Tokuhiro, K., Nagamori, E., Kitamoto, K., Takahashi, H. (2005)** Genetically engineered wine yeast produces a high concentration of L-lactic acid of extremely high optical purity. Appl. Environ. Microbiol. 71(5):2789-92.
- Sambrook, J., Fritsch, E. F., Maniatis, T. (1989)** Molecular cloning: a laboratory manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y.
- Sauer, M., Porro, D., Mattanovich, D., Branduardi, P. (2008)** Microbial production of organic acids: expanding the markets. Trends Biotechnol. 26(2):100-8.
- Sauer, U., and Eikmanns, B.J. (2005)** The PEP-pyruvate-oxaloacetate node as the switch point for carbon flux distribution in bacteria. FEMS Microbiol. Rev. 29(4):765-94.
- Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., Pühler, A. (1994)** Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69-73
- Schlösser, A., Hamann, A., Bossemeyer, D., Schneider, E., Bakker, E.P. (1993)** NAD⁺ binding to the *Escherichia coli* K⁺-uptake protein TrkA and sequence similarity between

- TrkA and domains of a family of dehydrogenases suggest a role for NAD⁺ in bacterial transport. *Mol. Microbiol.* 9(3):533-43.
- Schultz, C., Niebisch, A., Gebel, L., Bott, M. (2007)** Glutamate production by *Corynebacterium glutamicum*: dependence on the oxoglutarate dehydrogenase inhibitor protein OdhI and protein kinase PknG. *Appl Microbiol Biotechnol.* 76(3):691-700.
- Seol, W. and Shatkin, A.J. (1991)** *Escherichia coli kgtP* encodes an alpha-ketoglutarate transporter. *Proc. Natl. Acad. Sci. U S A.* 88(9):3802-6.
- Seol, W. and Shatkin, A.J. (1992)** *Escherichia coli* alpha-ketoglutarate permease is a constitutively expressed proton symporter. *J. Biol. Chem.* 267(9):6409-13.
- Silberbach, M., Schäfer, M., Hüser, A.T., Kalinowski, J., Pühler, A., Krämer, R., Burkovski, A. (2005)** Adaptation of *Corynebacterium glutamicum* to ammonium limitation: a global analysis using transcriptome and proteome techniques. *Appl. Environ. Microbiol.* 71(5):2391-402.
- Simic P., Sahm H., Eggeling L. (2001)** L-threonine export: use of peptides to identify a new translocator from *Corynebacterium glutamicum*. *J. Bacteriol.* 183(18):5317-24.
- Six, S., Andrews, S.C., Uden, G., Guest, J.R. (1994)** *Escherichia coli* possesses two homologous anaerobic C₄-dicarboxylate membrane transporters (DcuA and DcuB) distinct from the aerobic dicarboxylate transport system (Dct). *J. Bacteriol.* 176(21):6470-8.
- Soares-Silva, I., Schuller, D., Andrade, R.P., Baltazar, F., Cássio, F., Casal, M. (2003)** Functional expression of the lactate permease Jen1p of *Saccharomyces cerevisiae* in *Pichia pastoris*. *Biochem. J.* 376(Pt 3):781-7.
- Stackebrandt, E., Rainey, F.A., and Ward-Rainey, N.L. (1997)** Proposal for a new hierarchic classification system, Actinobacteria classis nov. *Int. J. Syst. Bacteriol.* 47, 479-491.
- Stansen, C., Uy, D., Delaunay, S., Eggeling, L., Goergen, J.L., Wendisch, V.F. (2005)** Characterization of a *Corynebacterium glutamicum* lactate utilization operon induced during temperature-triggered glutamate production. *Appl. Environ. Microbiol.* 71(10):5920-8.
- Steffgen, J., Burckhardt, B.C., Langenberg, C., Kühne, L., Müller, G.A., Burckhardt, G., Wolff, N.A. (1999)** Expression cloning and characterization of a novel sodium-dicarboxylate cotransporter from winter flounder kidney. *J. Biol. Chem.* 274(29):20191-6.
- Suwannakham, S., Huang, Y., Yang, S.T. (2006)** Construction and characterization of *ack* knock-out mutants of *Propionibacterium acidipropionici* for enhanced propionic acid fermentation. *Biotechnol. Bioeng.* 94(2):383-95.
- Tauch, A., Kirchner, O., Löffler, B., Götter, S., Pühler, A., Kalinowski, J. (2002)** Efficient electrotransformation of *Corynebacterium diphtheriae* with a minireplicon derived from the *Corynebacterium glutamicum* plasmid pGA1. *Curr. Microbiol.* 45: 362-367.

- Tauch, A., Krieft, S., Pühler, A., Kalinowski, J. (1999)** The *tetAB* genes of the *Corynebacterium striatum* R-plasmid pTP10 encode an ABC transporter and confer tetracycline, oxytetracycline and oxacillin resistance in *Corynebacterium glutamicum*. FEMS Microbiol. Lett. 173(1):203-9.
- Teramoto, H., Shirai, T., Inui, M., Yukawa, H. (2008)** Identification of a gene encoding a transporter essential for utilization of C4-dicarboxylates in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. (Epub ahead of print)
- Thomas, G.H., Southworth, T., León-Kempis, M.R., Leech, A., Kelly, D.J. (2006)** Novel ligands for the extracellular solute receptors of two bacterial TRAP transporters. Microbiology. 152(Pt 1):187-98.
- Trötschel, C. (2005)** Methioninaufnahme und –export in *Corynebacterium glutamicum*. PhD thesis, University of Cologne.
- Trötschel, C., Follmann, M., Nettekoven, J.A., Mohrbach, T., Forrest, L., Burkovski, A., Marin, K., Krämer, R. (2008)** Methionine uptake in *Corynebacterium glutamicum* by MetQNI and by MetP, a novel methionine and alanine importer of the NSS neurotransmitter transporter family. Submitted.
- van Maris, A.J., Winkler, A.A., Porro, D., van Dijken, J.P., Pronk, J.T. (2004a)** Homofermentative lactate production cannot sustain anaerobic growth of engineered *Saccharomyces cerevisiae*: possible consequence of energy-dependent lactate export. Appl. Environ. Microbiol. 70(5):2898-905.
- van Maris, A.J., Konings, W.N., van Dijken, J.P., Pronk, J.T. (2004b)** Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes. Metab Eng. 6(4):245-55.
- Vrljic, M., Sahm, H., Eggeling, L. (1996)** A new type of transporter with a new type of cellular function: L-lysine export from *Corynebacterium glutamicum*. Mol. Microbiol. 22(5):815-26.
- Wadler, C.S., Vanderpool, C.K. (2007)** A dual function for a bacterial small RNA: SgrS performs base pairing-dependent regulation and encodes a functional polypeptide. Proc Natl Acad Sci U S A. 104(51):20454-9.
- Warner, J.B. and Lolkema, J.S. (2002)** Growth of *Bacillus subtilis* on citrate and isocitrate is supported by the Mg²⁺-citrate transporter CitM. Microbiology. 148(Pt 11):3405-12.
- Wei, Y., Guffanti, A.A., Ito, M., Krulwich, T.A. (2000)** *Bacillus subtilis* Yqkl is a novel malic/Na⁺-lactate antiporter that enhances growth on malate at low protonmotive force. J. Biol. Chem. 275(39):30287-92.
- Wendisch, V.F., de Graaf, A.A., Sahm, H., Eikmanns, B.J. (2000)** Quantitative determination of metabolic fluxes during cointilization of two carbon sources: comparative analyses with *Corynebacterium glutamicum* during growth on acetate and/or glucose. J. Bacteriol. 182(11):3088-96.

- Wendisch, V.F., Bott, M., Eikmanns, B.J. (2006)** Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for biotechnological production of organic acids and amino acids. *Curr. Opin. Microbiol.* 9(3):268-74.
- Wendisch, V.F. (2008)** DNA microarray-based transcriptome analysis in *C. glutamicum*. In *Corynebacteria: Genomics and molecular biology* (Burkovski A., ed.), Horizon Scientific Press, Norwich, UK, pp. 33-54.
- Wennerhold, J. and Bott, M. (2006)** The DtxR regulon of *Corynebacterium glutamicum*. *J. Bacteriol.* 188(8):2907-18.
- Wennerhold, J., Krug, A., Bott, M. (2005)** The AraC-type regulator RipA represses aconitase and other iron proteins from *Corynebacterium* under iron limitation and is itself repressed by DtxR. *J. Biol.Chem.* 280: 40500-40508.
- Winnen, B., Felce, J., Saier, M.H. (2005)** Genomic Analyses of Transporter Proteins in *Corynebacterium glutamicum* and *Corynebacterium efficiens*. In *Handbook of Corynebacterium glutamicum*, (Eggeling, L. and Bott, M. eds), pp. 305-332, CRC Press, Boca Raton, Florida, USA.
- Yanisch-Perron, C., Viera, J., Messing, J. (1985)** Improved M13 phage vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* 33: 103-119.
- Yokota, A. and Lindley, N.D. (2005)** Central metabolism: sugar uptake and conversion. In *Handbook of Corynebacterium glutamicum*, (Eggeling, L. and Bott, M. eds), pp. 215-240, CRC Press, Boca Raton, Florida, USA.
- Youn, J.W., Jolkver, E., Krämer, R., Marin, K., Wendisch, V.F. (2008)** Identification and characterization of the dicarboxylate uptake system DccT in *Corynebacterium glutamicum*. *J. Bacteriol.* (Epub ahead of print).
- Yurtsever, T. (2008)** Characterization of putative CIC chloride channel and sodium/proton antiporters of *Corynebacterium glutamicum*. Master thesis, University of Cologne.
- Zientz, E., Six, S., Unden, G. (1996)** Identification of a third secondary carrier (DcuC) for anaerobic C₄-dicarboxylate transport in *Escherichia coli*: roles of the three Dcu carriers in uptake and exchange. *J. Bacteriol.* 178(24):7241-7.
- Zientz, E., Janausch, I.G., Six, S., Unden, G. (1999)** Functioning of DcuC as the C₄-dicarboxylate carrier during glucose fermentation by *Escherichia coli*. *J. Bacteriol.* 181(12):3716-20.

7 Appendix

7.1 Bacterial strains

Bacterial strains used in the study are summarized in table 7.1.

Table 7.1 *E. coli* and *Corynebacterium* strains used in this work.

Strain	Genotype, phenotype	Reference
<i>E. coli</i>		
DH5 α mcr	<i>endA1 supE44 thi-1 λ^- recA1 GyrA96 relA1 deoR Δ(lacZYA-argF) U169 ϕ80ΔlacZ ΔM15mcrA Δ(mmr hsdRMS mcrBC)</i>	Grant <i>et al.</i> , 1990
JM109	<i>F' traD36 lacI^f Δ(lacZ)M15 proA⁺B⁺ I e14⁺ (McrA⁻) Δ(lac-proAB) thi gyrA96 (Nx^R) endA1 hsdR17 (<i>r_km_k</i>) relA1 supE44 recA1</i>	Yanisch-Perron <i>et al.</i> , 1985
<i>C. efficiens</i>		
DSM 44549	Former YS-314, isolated from soil	Fudou <i>et al.</i> , 2002
<i>C. glutamicum</i>		
ATCC 13032	wild type	Abe <i>et al.</i> , 1967
RES167	ATCC 13032 Δ (<i>cglIIM-cglIRR-cglIIR</i>)	Tauch <i>et al.</i> , 2002
MSM	Spontaneous mutant of ATCC 13032 isolated on malate	Youn, personal communication
SSM	Spontaneous mutant of ATCC 13032 isolated on succinate	Youn <i>et al.</i> , 2008
FSM	Spontaneous mutant of ATCC 13032 isolated on fumarate	Youn <i>et al.</i> , 2008
pVWEx1- <i>dccT</i>	expression of <i>dccT</i> , Km ^R	Youn <i>et al.</i> , 2008
pVWEx1- <i>dctA</i>	expression of <i>dctA</i> , Km ^R	Youn, personal communication
Δ <i>mctC</i> pVWEx1- <i>dccT</i>	deletion of <i>mctC</i> , expression of <i>dccT</i> , Km ^R	This work
Δ <i>mctC</i> pVWEx1- <i>dctA</i>	deletion of <i>mctC</i> , expression of <i>dctA</i> , Km ^R	This work
icgI0028	pDrive insertion in <i>cglI0028</i> , Km ^R	This work
icgI0067	pDrive insertion in <i>cglI0067</i> , Km ^R	This work
icgI0092	pDrive insertion in <i>cglI0092</i> , Km ^R	Boltres, personal communication
icgI0101	pDrive insertion in <i>cglI0101</i> , Km ^R	This work
icgI0110	pDrive insertion in <i>cglI0110</i> , Km ^R	This work
icgI0146	pDrive insertion in <i>cglI0146</i> , Km ^R	Boltres, personal communication
icgI0206	pDrive insertion in <i>cglI0206</i> , Km ^R	This work
icgI0224	pDrive insertion in <i>cglI0224</i> , Km ^R	This work
icgI0225	pDrive insertion in <i>cglI0225</i> , Km ^R	This work
icgI0231	pDrive insertion in <i>cglI0231</i> , Km ^R	Boltres, personal communication
icgI0250	pDrive insertion in <i>cglI0250</i> , Km ^R	This work

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icg/0267	pDrive insertion in <i>cg/0267</i> , Km ^R	This work
icg/0283	pDrive insertion in <i>cg/0283</i> , Km ^R	This work
icg/0322	pDrive insertion in <i>cg/0322</i> , Km ^R	Mohrbach, 2006
icg/0324	pDrive insertion in <i>cg/0324</i> , Km ^R	This work
icg/0421	pDrive insertion in <i>cg/0421</i> , Km ^R	Mohrbach, 2006
icg/0470	pDrive insertion in <i>cg/0470</i> , Km ^R	Trötschel, 2005
icg/0481	pDrive insertion in <i>cg/0481</i> , Km ^R	Mohrbach, 2006
icg/0590	pDrive insertion in <i>cg/0590</i> , Km ^R	Becker, 2007
icg/0605	pDrive insertion in <i>cg/0605</i> , Km ^R	This work
icg/0635	pDrive insertion in <i>cg/0635</i> , Km ^R	Trötschel, 2005
icg/0640	pDrive insertion in <i>cg/0640</i> , Km ^R	This work
icg/0670	pDrive insertion in <i>cg/0670</i> , Km ^R	This work
icg/0679	pDrive insertion in <i>cg/0679</i> , Km ^R	This work
icg/0833	pDrive insertion in <i>cg/0833</i> , Km ^R	Ballan, 2007
icg/0850	pDrive insertion in <i>cg/0850</i> , Km ^R	This work
icg/0870	pDrive insertion in <i>cg/0870</i> , Km ^R	This work
icg/0912	pDrive insertion in <i>cg/0912</i> , Km ^R	Ballan, 2007
icg/0924	pDrive insertion in <i>cg/0924</i> , Km ^R	Boltres, personal communication
icg/0930	pDrive insertion in <i>cg/0930</i> , Km ^R	This work
icg/0951	pDrive insertion in <i>cg/0951</i> , Km ^R	This work
icg/0964	pDrive insertion in <i>cg/0964</i> , Km ^R	This work
icg/0968	pDrive insertion in <i>cg/0968</i> , Km ^R	Trötschel, 2005
icg/1005	pDrive insertion in <i>cg/1005</i> , Km ^R	This work
icg/1010	pDrive insertion in <i>cg/1010</i> , Km ^R	This work
icg/1061	pDrive insertion in <i>cg/1061</i> , Km ^R	Ballan, 2007
icg/1076	pDrive insertion in <i>cg/1076</i> , Km ^R	Ballan, 2007
icg/1080	pDrive insertion in <i>cg/1080</i> , Km ^R	Boltres, personal communication
icg/1082	pDrive insertion in <i>cg/1082</i> , Km ^R	This work
icg/1088	pDrive insertion in <i>cg/1088</i> , Km ^R	This work
icg/1107	pDrive insertion in <i>cg/1107</i> , Km ^R	Trötschel, 2005
icg/1148	pDrive insertion in <i>cg/1148</i> , Km ^R	This work
icg/1155	pDrive insertion in <i>cg/1155</i> , Km ^R	Trötschel, 2005
icg/1163	pDrive insertion in <i>cg/1163</i> , Km ^R	This work
icg/1252	pDrive insertion in <i>cg/1252</i> , Km ^R	Ballan, 2007
icg/1258	pDrive insertion in <i>cg/1258</i> , Km ^R	This work
icg/1262	pDrive insertion in <i>cg/1262</i> , Km ^R	This work
icg/1270	pDrive insertion in <i>cg/1270</i> , Km ^R	Krämer, personal communication
icg/1281	pDrive insertion in <i>cg/1281</i> , Km ^R	This work
icg/1331	pDrive insertion in <i>cg/1331</i> , Km ^R	Trötschel, 2005
icg/1386	pDrive insertion in <i>cg/1386</i> , Km ^R	This work

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icg/1420	pDrive insertion in <i>cgl/1420</i> , Km ^R	Boltres, personal communication
icg/1436	pDrive insertion in <i>cgl/1436</i> , Km ^R	This work
icg/1460	pDrive insertion in <i>cgl/1460</i> , Km ^R	This work
icg/1502	pDrive insertion in <i>cgl/1502</i> , Km ^R	This work
icg/1522	pDrive insertion in <i>cgl/1522</i> , Km ^R	This work
icg/1524	pDrive insertion in <i>cgl/1524</i> , Km ^R	Boltres, personal communication
icg/1525	pDrive insertion in <i>cgl/1525</i> , Km ^R	This work
icg/1554	pDrive insertion in <i>cgl/1554</i> , Km ^R	Ballan, 2007
icg/1688	pDrive insertion in <i>cgl/1688</i> , Km ^R	Boltres, personal communication
icg/1982	pDrive insertion in <i>cgl/1982</i> , Km ^R	This work
icg/1993	pDrive insertion in <i>cgl/1993</i> , Km ^R	This work
icg/2004	pDrive insertion in <i>cgl/2004</i> , Km ^R	This work
icg/2018	pDrive insertion in <i>cgl/2018</i> , Km ^R	Boltres, personal communication
icg/2045	pDrive insertion in <i>cgl/2045</i> , Km ^R	This work
icg/2077	pDrive insertion in <i>n cgl/2077</i> , Km ^R	Ballan, 2007
icg/2089	pDrive insertion in <i>cgl/2089</i> , Km ^R	This work
icg/2131	pDrive insertion in <i>cgl/2131</i> , Km ^R	This work
icg/2145	pDrive insertion in <i>cgl/2145</i> , Km ^R	Boltres, personal communication
icg/2211	pDrive insertion in <i>cgl/2211</i> , Km ^R	This work
icg/2250	pDrive insertion in <i>cgl/2250</i> , Km ^R	Boltres, personal communication
icg/2314	pDrive insertion in <i>cgl/2314</i> , Km ^R	This work
icg/2318	pDrive insertion in <i>cgl/2318</i> , Km ^R	This work
icg/2323	pDrive insertion in <i>cgl/2323</i> , Km ^R	Ballan, 2007
icg/2326	pDrive insertion in <i>cgl/2326</i> , Km ^R	This work
icg/2327	pDrive insertion in <i>cgl/2327</i> , Km ^R	This work
icg/2338	pDrive insertion in <i>cgl/2338</i> , Km ^R	This work
icg/2344	pDrive insertion in <i>cgl/2344</i> , Km ^R	Boltres, personal communication
icg/2385	pDrive insertion in <i>cgl/2385</i> , Km ^R	This work
icg/2386	pDrive insertion in <i>cgl/2386</i> , Km ^R	This work
icg/2460	pDrive insertion in <i>cgl/2460</i> , Km ^R	Trötschel, 2005
icg/2499	pDrive insertion in <i>cgl/2499</i> , Km ^R	This work
icg/2512	pDrive insertion in <i>cgl/2512</i> , Km ^R	This work
icg/2551	pDrive insertion in <i>cgl/2551</i> , Km ^R	This work
icg/2572	pDrive insertion in <i>cgl/2572</i> , Km ^R	This work
icg/2595	pDrive insertion in <i>cgl/2595</i> , Km ^R	This work
icg/2629	pDrive insertion in <i>cgl/2629</i> , Km ^R	This work
icg/2637	pDrive insertion in <i>cgl/2637</i> , Km ^R	Mohrbach, 2006
icg/2654	pDrive insertion in <i>cgl/2654</i> , Km ^R	This work
icg/2685	pDrive insertion in <i>cgl/2685</i> , Km ^R	Boltres, personal communication
icg/2731	pDrive insertion in <i>cgl/2731</i> , Km ^R	This work

<i>icg12744</i>	pDrive insertion in <i>cgl12744</i> , Km ^R	This work
<i>icg12746</i>	pDrive insertion in <i>cgl12746</i> , Km ^R	Boltres, personal communication
<i>icg12831</i>	pDrive insertion in <i>cgl12831</i> , Km ^R	This work
<i>icg12858</i>	pDrive insertion in <i>cgl12858</i> , Km ^R	This work
<i>icg12867</i>	pDrive insertion in <i>cgl12867</i> , Km ^R	Boltres, personal communication
<i>icg12908</i>	pDrive insertion in <i>cgl12908</i> , Km ^R	This work
<i>icg12912</i>	pDrive insertion in <i>cgl12912</i> , Km ^R	This work
<i>icg12917</i>	pDrive insertion in <i>cgl12917</i> , Km ^R	This work
<i>icg12929</i>	pDrive insertion in <i>cgl12929</i> , Km ^R	This work
<i>icg12942</i>	pDrive insertion in <i>cgl12942</i> , Km ^R	This work
<i>icg13027</i>	pDrive insertion in <i>cgl13027</i> , Km ^R	This work
<i>icg13042</i>	pDrive insertion in <i>cgl13042</i> , Km ^R	This work
<i>icg13074</i>	pDrive insertion in <i>cgl13074</i> , Km ^R	This work
<i>icg13083</i>	pDrive insertion in <i>cgl13083</i> , Km ^R	This work
<i>ldhA</i>	pDrive insertion in <i>ldhA</i> , Km ^R	This work
$\Delta aceE$	deletion of <i>aceE</i>	Blombach <i>et al.</i> , 2007a
$\Delta mctC$	deletion of <i>mctC</i>	This work
Δpgi	deletion of <i>pgi</i>	Hagmann, 2007

7.2 Plasmids

Plasmids used in this study are listed in table 7.2.

Table 7.2 Plasmids used in this work.

Plasmid	Description	Reference
pDrive	Ap ^R , Km ^R , <i>lacZ</i> α , A-T insertion vector	Qiagen, Hilden, Germany
pK18 <i>mobsacB</i>	Km ^R , ori pUC, <i>mob</i> , <i>sacB</i>	Schäfer <i>et al.</i> , 1994
pVWEx1	Km ^R , <i>lacI</i> ^f , <i>ptac</i> , <i>E. coli</i> - <i>C. glutamicum</i> shuttle vector	Peters-Wendisch <i>et al.</i> , 2001
pDrive-0028	pDrive with internal fragment of <i>cgl0028</i>	This work
pDrive-0067	pDrive with internal fragment of <i>cgl0067</i>	Follmann, 2004
pDrive-0101	pDrive with internal fragment of <i>cgl0101</i>	This work
pDrive-0110	pDrive with internal fragment of <i>cgl0110</i>	Follmann, 2004
pDrive-0206	pDrive with internal fragment of <i>cgl0206</i>	Follmann, 2004
pDrive-0224	pDrive with internal fragment of <i>cgl0224</i>	This work
pDrive-0225	pDrive with internal fragment of <i>cgl0225</i>	Follmann, 2004
pDrive-0250	pDrive with internal fragment of <i>cgl0250</i>	This work
pDrive-0267	pDrive with internal fragment of <i>cgl0267</i>	This work
pDrive-0283	pDrive with internal fragment of <i>cgl0283</i>	This work
pDrive-0324	pDrive with internal fragment of <i>cgl0324</i>	This work

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pDrive-0605	pDrive with internal fragment of <i>cgl0605</i>	Follmann, 2004
pDrive-0640	pDrive with internal fragment of <i>cgl0640</i>	This work
pDrive-0670	pDrive with internal fragment of <i>cgl0670</i>	This work
pDrive-0679	pDrive with internal fragment of <i>cgl0679</i>	This work
pDrive-0833	pDrive with internal fragment of <i>cgl0833</i>	Follmann, 2004
pDrive-0850	pDrive with internal fragment of <i>cgl0850</i>	This work
pDrive-0870	pDrive with internal fragment of <i>cgl0870</i>	This work
pDrive-0930	pDrive with internal fragment of <i>cgl0930</i>	This work
pDrive-0951	pDrive with internal fragment of <i>cgl0951</i>	This work
pDrive-0964	pDrive with internal fragment of <i>cgl0964</i>	This work
pDrive-1005	pDrive with internal fragment of <i>cgl1005</i>	Follmann, 2004
pDrive-1010	pDrive with internal fragment of <i>cgl1010</i>	This work
pDrive-1082	pDrive with internal fragment of <i>cgl1082</i>	This work
pDrive-1088	pDrive with internal fragment of <i>cgl1088</i>	This work
pDrive-1148	pDrive with internal fragment of <i>cgl1148</i>	This work
pDrive-1163	pDrive with internal fragment of <i>cgl1163</i>	Follmann, 2004
pDrive-1258	pDrive with internal fragment of <i>cgl1258</i>	Follmann, 2004
pDrive-1262	pDrive with internal fragment of <i>cgl1262</i>	This work
pDrive-1281	pDrive with internal fragment of <i>cgl1281</i>	This work
pDrive-1386	pDrive with internal fragment of <i>cgl1386</i>	This work
pDrive-1436	pDrive with internal fragment of <i>cgl1436</i>	Follmann, 2004
pDrive-1460	pDrive with internal fragment of <i>cgl1460</i>	This work
pDrive-1502	pDrive with internal fragment of <i>cgl1502</i>	Follmann, 2004
pDrive-1522	pDrive with internal fragment of <i>cgl1522</i>	This work
pDrive-1525	pDrive with internal fragment of <i>cgl1525</i>	This work
pDrive-1982	pDrive with internal fragment of <i>cgl1982</i>	This work
pDrive-1993	pDrive with internal fragment of <i>cgl1993</i>	This work
pDrive-2004	pDrive with internal fragment of <i>cgl2004</i>	This work
pDrive-2045	pDrive with internal fragment of <i>cgl2045</i>	Follmann, 2004
pDrive-2089	pDrive with internal fragment of <i>cgl2089</i>	This work
pDrive-2131	pDrive with internal fragment of <i>cgl2131</i>	This work
pDrive-2211	pDrive with internal fragment of <i>cgl2211</i>	This work
pDrive-2314	pDrive with internal fragment of <i>cgl2314</i>	This work
pDrive-2318	pDrive with internal fragment of <i>cgl2318</i>	This work
pDrive-2326	pDrive with internal fragment of <i>cgl2326</i>	This work
pDrive-2327	pDrive with internal fragment of <i>cgl2327</i>	Follmann, 2004
pDrive-2338	pDrive with internal fragment of <i>cgl2338</i>	Follmann, 2004
pDrive-2385	pDrive with internal fragment of <i>cgl2385</i>	This work
pDrive-2386	pDrive with internal fragment of <i>cgl2386</i>	This work
pDrive-2499	pDrive with internal fragment of <i>cgl2499</i>	This work

pDrive-2512	pDrive with internal fragment of <i>cgl2512</i>	This work
pDrive-2551	pDrive with internal fragment of <i>cgl2551</i>	Follmann, 2004
pDrive-2572	pDrive with internal fragment of <i>cgl2572</i>	This work
pDrive-2595	pDrive with internal fragment of <i>cgl2595</i>	Follmann, 2004
pDrive-2629	pDrive with internal fragment of <i>cgl2629</i>	This work
pDrive-2654	pDrive with internal fragment of <i>cgl2654</i>	This work
pDrive-2731	pDrive with internal fragment of <i>cgl2731</i>	This work
pDrive-2744	pDrive with internal fragment of <i>cgl2744</i>	Follmann, 2004
pDrive-2831	pDrive with internal fragment of <i>cgl2831</i>	This work
pDrive-2858	pDrive with internal fragment of <i>cgl2858</i>	This work
pDrive-2908	pDrive with internal fragment of <i>cgl2908</i>	Follmann, 2004
pDrive-2912	pDrive with internal fragment of <i>cgl2912</i>	This work
pDrive-2917	pDrive with internal fragment of <i>cgl2917</i>	This work
pDrive-2929	pDrive with internal fragment of <i>cgl2929</i>	This work
pDrive-2942	pDrive with internal fragment of <i>cgl2942</i>	This work
pDrive-3027	pDrive with internal fragment of <i>cgl3027</i>	Follmann, 2004
pDrive-3042	pDrive with internal fragment of <i>cgl3042</i>	Follmann, 2004
pDrive-3074	pDrive with internal fragment of <i>cgl3074</i>	Follmann, 2004
pDrive-3083	pDrive with internal fragment of <i>cgl3083</i>	This work
pDrive- <i>ldhA</i>	pDrive with internal fragment of <i>ldhA</i>	This work
pK18 <i>mobsacB</i> -0832-0833	pK18 <i>mobsacB</i> with flanking regions of <i>cgl0832-0833</i>	This work
pVWEx1- <i>dccT</i>	pVWEx1 with complete <i>dccT</i> sequence	Youn et al., 2008
pVWEx1- <i>dctA</i>	pVWEx1- with complete <i>dctA</i> sequence	Youn, personal communication

Ap^R, resistance towards ampicillin. Km^R, resistance towards kanamycin

7.3 Oligonucleotides

The oligonucleotides used in this work are listed in table 7.3.

Table 7.3 Oligonucleotides used in this work. Restriction enzymes recognition sites are indicated by minuscules and preceded by a short random sequence.

Oligonucleotide	Sequence (5'-3')	Reference
0028ins_F	TATGTCGCACGGCTTGCT	This work
0028ins_R	TTCCGCGCATCCAAGAAG	This work
i_Cgl0101_5'	CGACGAGCAAGCTCAGTA	This work
i_Cgl0101_3'	GCCCATCATGTTTACGAC	This work
Cgl0224_F	ACTCCAGGCTGATCTCAT	This work
Cgl0224_R	CAACGAGGTCCAACCTGTA	This work
Cgl0250_F	CAGTCCACGACTTGGAAAT	This work
Cgl0250_R	TCAATGGCTACCGACTAC	This work
Cgl0267_F	AGGCTGGCGATGATGATG	This work
Cgl0267_R	AACGGTGCTCTGCTGACT	This work

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i_Cgl0283_5'	ACTTCGGTGACCGTCTTG	This work
i_Cgl0283_3'	GATGGTGAATGCCGAGAG	This work
Cgl0324_F	AACGCAGCTCTTGTTTC	This work
Cgl0324_R	CGGCTTCGTAGTATTC	This work
Cgl0640_F	GCTGCCGATTCTATTGAC	This work
Cgl0640_R	TAGTGATAAGGCGGTGCT	This work
0670_F	CATATCGGCGGACATATC	This work
0670_R	TAGGCCATCAGCACAATC	This work
Cgl0679_F	GCGATCTTTCGCTTAGAAC	This work
Cgl0679_R	TACCGGAGACAATTCCAC	This work
Cgl0833F	CAGAACGGTCTGGCTATC	Follmann, 2004
Cgl0833R	ATTGCCCAGTCTGCACCT	Follmann, 2004
Cgl0850_F	CCGCCTCTAATAACGAAG	This work
Cgl0850_R	GTTGATGGTCGGTACTGT	This work
0870ins_F	CTTCGGCGGTTCCAATGT	This work
0870ins_R	ATGCGTCGCAGCATGAAG	This work
i_Cgl0930_5'	ATGCGTTGCAGCCGTGTT	This work
i_Cgl0930_3'	GCCGTAGAGGTCCTTGAA	This work
Cgl0951_F	ACAGCCTCAGGAACAT	This work
Cgl0951_R	TCCGGAACGTACTGAT	This work
0964ins_F	GGCGCAATCAATCACCTC	This work
0964ins_R	ACAGCTTCAGCGGCTCAA	This work
Cgl1010_F	CTCAATGCCATCAACGAG	This work
Cgl1010_R	ACCGAAGCTGCTGTCAAT	This work
Cgl1082_F	CGTGGCAGTCATCCTATT	This work
Cgl1082_R	CATGCACAGCACTGACTT	This work
1088ins_F	ATTAGACGATGTCGCAGC	This work
1088ins_R	TCCAACGACCATGATGTG	This work
1148ins_F	GGTGATAATCGCAGGTGT	This work
1148ins_R	AATGCGACCGATAGTGTG	This work
1262ins_F	GTCATCACAAC TGCCACG	This work
1262ins_R	ATTGCGGTTCTTCTCGTG	This work
Cgl1281_5	GATCTTCAGCAGCGCAGTTA	This work
Cgl1281_3	GGAATGCTCCAACGCATATTG	This work
1386ins_F	CGGCGAGTTCTTAGTTCT	This work
1386ins_R	CTCTTCAGCACCGACAAC	This work
1460ins_F	CATGTGTCGGTGACGTAT	This work
1460ins_R	CACAAGCCTTGTTGGTGTA	This work
i_Cgl1522_5'	AGTTCAGCATCGGGAAGA	This work
i_Cgl1522_3'	GCACGGCGGATAACCAAA	This work
Cgl1525_F	CAATCGGCTCAAGATGTC	This work
Cgl1525_R	GGCTTATGCGTCACTGTT	This work
Cgl1982_F	CCAGACGCCTGGTAAGAA	This work
Cgl1982_R	GGAAC TACCGCGAGATCA	This work
Cgl1933_5	GCAAGCGCGGTGTGGATTAC	This work
Cgl1933_3	GAGCGGACAACGACCTTGGA	This work
Cgl2004_F	AACGACGTTGGTTCGTAG	This work
Cgl2004_R	TGAGAAGCCGATGAGAG	This work
Cgl2089_F	CAGAGCGAACTGCCTCAT	This work
Cgl2089_R	CCTCCAAGGACCGAAGAT	This work

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i_Cgl2131_5'	TCGGCGTACTCGCATACT	This work
i_Cgl2131_3'	GTGCCCCACTGCCATTGTT	This work
2211ins_F	TGACGGTCGGACTATTGC	This work
2211ins_R	AGCCGATGATGGTGAGTG	This work
i_Cgl2314_5'	CGTGGTCATTGCACTCTC	This work
i_Cgl2314_3'	GACATTCTGCGCTGTTGG	This work
Cgl2318_F	GTTGCTGTTGACGACAGT	This work
Cgl2318_R	TAGTGGATGCTGCTCAGT	This work
2326ins_F	CGCGACTTCTTCATCGAC	This work
2326ins_R	GCCACAGCATCCAATACC	This work
i_Cgl2385_5'	AAGGAGCCTGGCATGGAT	This work
i_Cgl2385_3'	CGCGATGGATGGAACAGA	This work
Cgl2386_F	GGTTATCGGCGTTGGATT	This work
Cgl2386_R	TAACAGCGGCGTAGAGGT	This work
2499ins_F	ACCCTGAGCGCAAGAAAC	This work
2499ins_R	AACCACACCTGCGATAGC	This work
2512ins_F	TGAAGCCGTGGAATAAGC	This work
2512ins_R	TCTTCGCTTTCAACGCTG	This work
2572ins_F	GTCCGATTTC AACAGAC	This work
2572ins_R	ACTACGGTGATTTCCACG	This work
2629ins_F	AATGATGCTGCGGTGGAT	This work
2629ins_R	TGGGTGACCACAACAACG	This work
i_Cgl2654_5'	ATTGTCGACGGCGTGAAC	This work
i_Cgl2654_3'	GCCTGCCACGAATTCCTT	This work
Cgl2731_F	CTTCGCAGTGTTGCTCGT	This work
Cgl2731_R	TTGCTAGTGCGGTTGCTC	This work
i_Cgl2831_5'	CGTGGTGCGCTCTAACTCA	This work
i_Cgl2831_3'	ATGGCAGGTCAGGATCAG	This work
2858ins_F	GCGGATTCATCAGCTCAC	This work
2858ins_R	GTGCCAGAGGAAGATGGA	This work
2912_F	GTTACGGCTGCTAACCTG	This work
2912_R	CCTGGTTACCGTGATGAT	This work
Cgl2917_F	GACAACCGCAGTAGATCA	This work
Cgl2917_R	GGATGCACAGATCCTGTA	This work
Cgl2929_F	ACCATGACGGAAGAGTTG	This work
Cgl2929_R	AACCAGCACTTCACTCAG	This work
Cgl2942_F	CTTCCGAGTGAAGAACTG	This work
Cgl2942_R	GTCTAGCTGGTTGGATTG	This work
i_Cgl3083_5'	GCGGAATCGGTGAGACTT	This work
i_Cgl3083_3'	CCACCGCGAATAATGGCA	This work
ldhA_F	GGACTGGAAGTTTCACTGT	This work
ldhA_R	GGAGTTGCATACGCATAC	This work
IS6100_F	CGCTGGTATTGTGCTAT	Mormann <i>et al.</i> , 2006
IS6100_R	CACCGGCTTGATCAGTAT	Mormann <i>et al.</i> , 2006
s6100e	GCGCCTTGTTGAGAGAGCTT	Mormann <i>et al.</i> , 2006
s6100x	CGGATAGCGACAATACCAGC	Mormann <i>et al.</i> , 2006
del0832l_F1_F	CGATActgcagCGAATGTCTCTGCATACC	This work
del0832l_F1_R	GCGCGCtctagaCACTGGAACTTATCTCG	This work
del0833_F2_F	GCGCGCtctagaCACTAAATCTAGTTTCTG	This work
del0833_F2_R	CGAtCGaagctTACGCCCTCATACAAG	This work

Fcgl0832/33ue_ej-70	GCatcctgcaggGGCATTGCTTAGAGAG	This work
Fcgl0833ue_ej	GCatcctgcaggTCAGATATGAATTCCAC	This work
Rcgl0832-0833ueberex2	GCGCTAGCGTGATCAACAGCctt	This work
M13 Universal -21	ACGACGTTGTAAAACGACGGCCAG	ZMMK, Cologne, Germany
M13 reverse	TTACACAGGAAACAGCTATGACC	ZMMK, Cologne, Germany

7.4 Complete list of the results of the DNA microarray analysis

Table 7.4 *C. glutamicum* genes that were found to be differentially expressed in a DNA microarray assay upon the application of 1-aminoethylphosphinate (AEP). The transcript ratio was calculated 10 min after AEP application and before. Of the detected genes, only those with a transcript ratio ≥ 1.5 and ≤ 0.7 are given. The number of encoded transmembrane helices (TMH) was predicted with TMHMM v.2.0 (Krogh *et al.*, 2001). The gene length is given in bp.

CDS	gene	gene product or deduced function	ratio	TMH	gene length
Upregulated genes					
Information storage and processing					
cgl0759		Putative ribosome-associated protein Y (PSrp-1)	3.6	0	665
cgl2279		Putative transcriptional regulator, ArsR-family	2.3	0	371
cgl1444		Putative transcriptional regulator, MerR-family	2.1	0	575
cgl2026	rpsB	30S ribosomal protein S2	1.9	0	818
cgl2115		Putative transcriptional regulator, ArsR-family	1.9	0	443
cgl0982	ripA	Putative transcriptional regulator, AraC-family	1.9	0	995
cgl1259	gatB	Glutamyl-tRNA (Gln) amidotransferase, subunit B	1.9	0	1481
cgl0254	sigC	RNA polymerase sigma factor, ECF-family	1.7	0	581
Cellular processes and signaling					
cgl1714		Putative secreted hydrolase	3.0	0	995
cgl1441		Putative signal transduction protein, FHA-domain	2.2	0	431
cgl0628		Putative secreted lipoprotein	1.9	1	650
cgl0033		Conserved hypothetical protein	1.8	1	812
cgl2532	nrdH	Conserved hypothetical protein, glutaredoxin-like protein NrdH	1.8	0	233
cgl1524	tetB	ABC-type multidrug transport system, ATPase and permease subunit	1.6	6	1799
cgl1753		Hypothetical protein, contains peptidoglycan-binding LysM domain	1.6	1	578
cgl1086	tpx	Thiol peroxidase	1.6	0	497
cgl2411	clpP2	Endopeptidase Clp, proteolytic subunit	1.5	0	626
cgl0325		Putative glycosyl transferase	1.5	0	710
Metabolism					
cgl0248	leuA	2-Isopropylmalate synthase	10.6	0	68
cgl0708	dtsR1	Acetyl/propionyl-CoA carboxylase, beta chain	2.8	1	1631
cgl0810		ABC-type putative iron-siderophore transporter, substrate-binding lipoprotein	2.2	0	1016
cgl2911	ldh	L-Lactate dehydrogenase	2.1	0	944
cgl2610	pqo	Pyruvate:quinone oxidoreductase	2.0	0	1739
cgl0709	birA	Bifunctional biotin-[acetyl-CoA-carboxylase] synthetase/biotin operon repressor	1.9	0	866
cgl2872	fadD2	Putative long-chain-fatty-acid-CoA ligase	1.8	0	1862
cgl2248	aceE	Pyruvate dehydrogenase (acetyl-transferring)	1.7	0	2768
cgl2390	pcaI	Putative 3-oxoadipate CoA-transferase	1.7	0	752
cgl2134		Putative oxidoreductase	1.7	0	887
cgl0086	ureC	Urease alpha subunit	1.7	0	1712
cgl2927	sod	Superoxide dismutase	1.7	0	602
cgl1223	ssuA	ABC-type aliphatic sulfonate transporter, substrate-binding	1.6	1	959

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		lipoprotein			
cgl2301	dcp	Peptidyl-dipeptidase	1.6	0	2036
cgl1395	argJ	Glutamate N-acetyltransferase	1.6	0	1166
cgl2531	nrdI	Conserved hypothetical protein, flavodoxin-like protein NrdI	1.6	0	446
cgl1687		ABC-type transporter, ATPase subunit	1.5	0	599
cgl2770	fda	Fructose-bisphosphate aldolase	1.5	0	1034
cgl1018	ssuD2	FMNH ₂ -dependent aliphatic sulfonate monooxygenase	1.5	0	1178
cgl1163	putP	Putative Na ⁺ /proline symporter, solute:sodium symporter (SSS) family	1.5	13	1574
Poorly characterized					
cgl1715		Putative secreted protein	3.6	1	377
cgl2564		Putative acetyltransferase	3.6	0	290
cgl0229		Conserved hypothetical protein	3.6	0	203
cgl1170		Conserved hypothetical protein	3.1	0	539
cgl2226		Hypothetical protein	3.1	0	545
cgl1652		Putative membrane protein	3.0	2	275
cgl0950		Hypothetical protein	2.8	0	134
cgl0950		Hypothetical protein	2.8	0	134
cgl1651		Putative secreted protein	2.5	0	557
cgl1294		Putative transcriptional regulator, HTH_3-family	2.5	0	215
cgl1135		Conserved hypothetical protein	2.5	0	803
cgl0679		Putative membrane protein	2.4	3	905
cgl2004		Putative secreted or membrane protein	2.4	2	347
cgl0062		Putative tautomerase	2.3	0	449
cgl0915		Conserved hypothetical protein	2.3	0	332
cgl1100	mshB	Putative N-acetyl-1-D-myo-inositol-2-amino-2-deoxy- α -D-glucopyranoside deacetylase	2.3	0	872
cgl1617		Hypothetical protein	2.2	0	188
cgl1096		Conserved hypothetical protein	2.1	0	539
cgl2227	hmuO	Heme oxygenase (decyclizing)	2.0	0	647
cgl0969		Hypothetical protein	2.0	0	212
cgl0041	pknB	Serine/threonine protein kinase	2.0	1	1940
cgl0840		Putative secreted protein	1.9	0	428
cgl0841		Hypothetical protein	1.9	0	269
cgl1832		Putative membrane protein	1.9	2	242
cgl2914		Conserved hypothetical protein, putative FMN reductase	1.9	0	545
cgl0969		Hypothetical protein	1.8	0	212
cgl1696		Putative secreted protein	1.8	0	737
cgl0525	dkg	Putative aldo/keto reductase, related to diketogulonate reductase	1.8	0	809
cgl1182		Putative secreted protein	1.7	1	122
cgl1114		Conserved hypothetical protein	1.7	0	167
cgl0751		Conserved hypothetical protein	1.7	0	353
cgl1615		Conserved hypothetical protein	1.7	0	479
cgl0343	cmt1	Trehalose corynomycyl transferase	1.7	0	1097
cgl1097		Putative secreted protein	1.7	0	716
cgl1374		Conserved hypothetical protein	1.7	0	914
cgl0791		Putative secreted protein	1.7	0	1064
cgl0955		Hypothetical protein	1.7	0	251
cgl2942		Putative membrane protein	1.7	3	263
cgl1241		Conserved hypothetical protein	1.7	0	992
cgl0105		ATP/GTP-binding protein	1.7	0	1955
cgl2005		Putative secreted or membrane protein	1.7	2	359
cgl2932		Conserved hypothetical protein	1.7	0	635
cgl3015		Putative secreted membrane protein	1.6	2	323
cgl1114		Conserved hypothetical protein	1.6	0	167
cgl1245		Conserved hypothetical protein	1.6	0	665

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cgl1235		Putative transcriptional regulator, HTH_3-family	1.6	0	227
cgl2677		Hypothetical protein, uncharacterized enzyme involved in biosynthesis of extracellular polysaccharides	1.6	0	293
cgl0395		Conserved hypothetical protein	1.6	0	815
cgl0768	whcE	Transcriptional regulator, WhiB-family	1.6	0	260
cgl3020		Putative plasmid maintenance system antidote protein	1.6	0	365
cgl1340		Hypothetical protein	1.6	0	221
cgl2075		Putative metal-binding, possibly nucleic acid-binding protein	1.6	0	536
cgl0324		Conserved putative membrane protein	1.6	3	377
cgl0373		Conserved putative membrane protein	1.6	2	338
cgl1766		Hypothetical protein	1.6	0	434
cgl2165		Putative secreted protein	1.5	1	530
cgl0796		Putative secreted protein	1.5	0	512
cgl2150		Cell division initiation protein - Antigen 84 homolog	1.5	0	1097
cgl2488		Putative membrane protein	1.5	1	275
cgl0805		Putative uroporphyrin-III C-methyltransferase	1.5	0	353
cgl2252		Conserved hypothetical protein	1.5	0	800
Downregulated genes					
Information storage and processing					
cgl0504	rpsJ	30S ribosomal protein S10	0.2	0	332
cgl0006	gyrB	DNA gyrase subunit B	0.3	0	2054
cgl0505	rplC	50S ribosomal protein L3	0.3	0	656
cgl0508	rplW	50S ribosomal protein L23	0.3	0	305
cgl1678		Putative N-acetyltransferase	0.3	0	569
cgl0507	rplD	50S ribosomal protein L4	0.3	0	656
cgl0510	rplB	50S ribosomal protein L2	0.4	0	842
cgl0174	cspA	Cold-shock protein A	0.4	0	203
cgl2037	rplS	50S ribosomal protein L19	0.4	0	341
cgl1378	infC	Translation initiation factor IF-3	0.4	0	569
cgl2363	rplU	50S ribosomal protein L21	0.4	0	305
cgl0867	rpsN	30S ribosomal protein S14	0.4	0	305
cgl0521	rplN	50S ribosomal protein L14	0.4	0	368
cgl1380	rplT	50S ribosomal protein L20	0.4	0	383
cgl1985	infB	Translation initiation factor 2 (GTPase)	0.4	0	3014
cgl0511	rpsS	30S ribosomal protein S19	0.4	0	278
cgl0560	infA	Translation initiation factor IF-1	0.4	0	218
cgl0523	rplE	50S ribosomal protein L5	0.4	0	575
cgl0540	rpsE	30S ribosomal protein S5	0.4	0	635
cgl0308	cspB	Cold-shock protein B	0.4	0	203
cgl0537	rpsH	30S ribosomal protein S8	0.5	0	398
cgl0566	rplQ	50S ribosomal protein L17	0.5	0	491
cgl0866	rpsR	30S ribosomal protein S18	0.5	0	251
cgl2362	rpmA	50S ribosomal protein L27	0.5	0	266
cgl0493	rpsL	30S ribosomal protein S12	0.5	0	368
cgl1976	rpsO	30S ribosomal protein S15.	0.5	0	269
cgl0522	rplX	50S ribosomal protein L24	0.5	0	314
cgl0539	rplR	50S ribosomal protein L18	0.5	0	404
cgl0513	rplV	50S ribosomal protein L22	0.5	0	362
cgl0542	rplO	50S ribosomal protein L15	0.5	0	446
cgl0538	rplF	50S ribosomal protein L6	0.5	0	536
cgl0517	rpsQ	30S ribosomal protein S17	0.5	0	278
cgl0562	rpsK	30S ribosomal protein S11	0.5	0	404
cgl0476	rplK	50S ribosomal protein L11	0.5	0	437
cgl1425	scpB	Putative segregation and condensation protein B	0.5	0	515
cgl0494	rpsG	30S ribosomal protein S7	0.6	0	467
cgl2054	rpsP	30S ribosomal protein S16	0.6	0	497

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cgl0561	rpsM	30S ribosomal protein S13	0.6	0	368
cgl1619	efp	Elongation factor P (EF-P).	0.6	0	563
cgl0515	rplP	50S ribosomal protein L16	0.6	0	416
cgl0488	rpoB	DNA-directed RNA polymerase, beta chain	0.6	0	3497
cgl0563	rpsD	30S ribosomal protein S4	0.6	0	605
cgl0514	rpsC	30S ribosomal protein S3	0.6	0	746
cgl0581	rplM	50S ribosomal protein L13	0.6	0	443
cgl1599	fmu	Ribosomal RNA small subunit methyltransferase B	0.6	0	1535
cgl0869	rpmB	50S ribosomal protein L28	0.6	0	236
cgl1199	rho	Transcription termination factor Rho	0.6	0	2288
cgl2560	ramA	Putative transcriptional regulator, LuxR-family, Nif-specific	0.6	0	845
cgl1240	trmU	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	0.6	0	1097
cgl0582	rpsI	30S ribosomal protein S9	0.6	0	548
cgl0564	rpoA	DNA-directed RNA polymerase, alpha subunit	0.6	0	1016
cgl0477	rplA	50S ribosomal protein L1	0.7	0	710
cgl0939	rplY	Ribosomal protein L25 (general stress protein Ctc)	0.7	0	602
Cellular processes and signaling					
cgl0783		Putative secreted protein, containing a PDZ-domain	0.3	1	1052
cgl0858		Putative secreted protein, related to metalloendopeptidases	0.4	1	713
cgl0344	wzz	Cell surface polysaccharide biosynthesis/chain length determinant	0.5	2	1436
cgl2188		Secreted protein NLP/P60 family	0.5	0	629
cgl1098		Putative GTPase, probably involved in stress response	0.6	0	1922
cgl1203		Putative membrane protein	0.6	11	1148
cgl0354		Putative glycosyltransferase	0.7	0	1037
Metabolism					
cgl2818	fpr2	Ferredoxin--NADP(+) reductase	0.3	0	1373
cgl1284	serA	Phosphoglycerate dehydrogenase	0.3	0	1592
cgl1609	carB	Carbamoyl-phosphate synthase, large chain	0.4	0	3341
cgl1129	odhA	2-Oxoglutarate dehydrogenase, E1 component	0.4	0	3773
cgl1401	argH	Argininosuccinate lyase	0.4	0	1433
cgl0996	glyA	Serine hydroxymethyltransferase	0.5	0	1304
cgl1316	leuD	3-Isopropylmalate dehydratase, small subunit	0.5	0	593
cgl0860	purN	Phosphoribosylglycinamide formyltransferase	0.6	0	632
cgl0607	guaA	Putative GMP synthase	0.6	0	1571
cgl1206	atpB	ATP synthase F0, A chain	0.6	6	968
cgl0031		ABC-type putative sugar transporter, permease subunit	0.6	10	1025
cgl0877		Putative molybdopterin biosynthesis protein	0.6	0	587
cgl0727		ABC-type putative sugar transporter, substrate-binding lipoprotein	0.6	0	1274
cgl1990		ABC-type putative dipeptide/oligopeptide transporter, substrate-binding lipoprotein	0.6	0	1604
Poorly characterized					
cgl0782		Conserved hypothetical protein	0.2	0	1448
cgl1379	rpmI	50S ribosomal protein L35	0.4	0	194
cgl2533	rpmJ	50S ribosomal protein L36	0.4	0	122
cgl1666		Putative membrane protein	0.4	1	464
cgl0819	rpf1	RPF-protein precursor	0.5	0	581
cgl1825		Hypothetical protein	0.5	0	212
cgl0541	rpmD	50S ribosomal protein L30	0.5	0	185
cgl0873	rpmF	50S ribosomal protein L32	0.5	0	173
cgl1471		Putative secreted protein	0.5	1	485
cgl1805		Hypothetical protein	0.6	0	308
cgl2721		Hypothetical protein	0.6	0	2456
cgl1688		ABC-type transporter, permease subunit	0.6	9	1001
cgl1908		Conserved hypothetical protein	0.6	0	293
cgl3099	rpmH	50S ribosomal protein L34	0.6	0	143

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cgl0868	rpmG	50S ribosomal protein L33	0.6	0	164
cgl0989		Hypothetical protein, similar to ribosomal protein S2	0.6	1	320
cgl1535		Conserved hypothetical protein	0.6	0	845
cgl0078		Putative secreted protein	0.6	0	560
cgl0413		Conserved hypothetical protein	0.6	0	101
cgl2343	rpsT	30S ribosomal protein S20	0.6	0	263
cgl1028		Putative membrane protein	0.6	8	1481
cgl1341	tnp23a	Transposase, putative pseudogene	0.6	0	194
cgl0516	rpmC	50S ribosomal protein L29	0.7	0	230
cgl2623		Conserved hypothetical protein	0.7	0	329

Danke...

...Herr Prof. Krämer für die Möglichkeit, ein so interessantes und abwechslungsreiches Thema zu bearbeiten, sowie für die sachlichen Besprechungen und ein reges Interesse am Fortschritt dieser Arbeit.

...Herr Prof. Flügge für die Übernahme des Koreferats und die Betreuung im Rahmen der IMPRS.

...Kay für die drei Jahre fruchtbarer Diskussionen, deinen Optimismus und Zuversicht, die besonders in Dürrezeiten den nötigen Gegenpol bildeten. Auch die ständige Bereitschaft, konstruktive Kritik zu üben, war maßgeblich für das Gelingen dieser Arbeit.

...liebe Kollegen, von denen ich die meisten inzwischen zu meinen guten Freunden zähle. Das hervorragende Arbeitsklima, ständige Hilfsbereitschaft, zahlreiche Späße und feierabendliche gemeinsame Stunden sorgten für eine permanent gute Laune und waren entscheidend für das Überstehen der harten Zeiten.

...Vera für die Unterstützung in wichtigen Entscheidungen, für unzählige interessante und wichtige Diskussionen und das tapfere Korrekturlesen der Arbeit. Die Sinnsuche ist nie vergebens!

...Phil für die anfängliche Lanze, Ausflippausen und die fernmündliche Unterstützung beim Schreiben.

...Nina für das Öffnen der Pandora box und die Schlafgelegenheit nach verpassten Zügen.

...Suey für das Training meiner Gesangkünste und die Aufbesserung des Englischen.

...Martin für deine gute Laune, die mir immer ein Grinsen entlockt hat und die Bioenergetikdiskussionen.

...Jeannine für die spannenden Klettergeschichten und die Tipps für *E. coli* Arbeiten.

...Jens für die Singstar-Abende und für die Tipps und Tricks auf dem Gebiet der Proteinforschung.

...Bettine für die nachahmenswerte positive Einstellung und die Unterstützung an der HPLC.

...Sascha für die gemeinsamen Geburtstagsfeiern und das geteilte Leid der Endphase.

...Alex, denn endlich kehrt die Normalität zu den Osmos zurück.

...Tobi für die Bereicherung meiner Musikenntnisse.

...Markus für den deftigen Humor und den entspannten Umgangston.

...Ines für die Modeberatung.

...Tina für die Unterstützung an der HPLC und bei den Screenings.

...Kirsten für unglaubliche Geschichten aus Australien.

...Gerd für Diskussionen über Coryne-Metabolismus.

...Anja, weil du immer alles wusstest und der Gute Geist des Labors bist.

...Ute für das glückliche Händchen im letzten Versuch.

...Eva, weil du auch ausgefallene Sachen zu finden wusstest.

...Astrid für manche psychologische Einsicht.

...Marc für hoffentlich wertvolle Karrieretipps.

...Gabi für Kolben, Medien und die große allgemeine Arbeitserleichterung.

...Catriona für die Einführung in die Mikroskopie.

...Frank und Inga für manch' lustige Geschichte.

...Jule und Maike für die Unterstützung in der Anfangsphase, Stefan und Eva für einige interessante Mutanten.

...Jung-Won Youn, Volker Wendisch, Denise Emer, Bernd Eikmanns, Christian Rückert und Jörn Kalinowski für die hervorragende Kooperation und somit einige Highlights der Arbeit, sowie Hans-Günther Schmalz und Janna Velder für die AEP-Synthese.

...*C. glutamicum* für die preisgegebenen Geheimnisse.

Ganz herzlicher Dank gilt meiner Mutter, die für meinen Werdegang maßgeblich entscheidend ist.

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. R. Krämer am Institut für Biochemie der Mathematisch-Naturwissenschaftlichen Fakultät der Universität zu Köln betreut worden.

Teilpublikationen:

Youn, J.W., Jolkver, E., Krämer, R., Marin, K., Wendisch, V.F. (2008) Identification and characterization of the dicarboxylate uptake system DccT in *Corynebacterium glutamicum*. J Bacteriol. (in press).

Jolkver, E., Emer, D., Ballan, S., Krämer, R., Eikmanns, B., Marin, K. (2008) Identification and characterization of a bacterial carrier system for the uptake of pyruvate, propionate and acetate in *Corynebacterium glutamicum*. Submitted.

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Datum: Köln, September 2008

Unterschrift:

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